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The relationship of magnesium, manganese, tryptophan and vitamin B6 to the immune response in rats

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INTRODUCTION

The relation between nutritional status and resistance to infectious disease has assumed increasing international importance as population growth continues to imperil adequacy of the world's food supply (Scrimshaw et al., 1968). Protein-calorie malnutrition is recognized as the major nutritional deficiency syndrome of the world (Caddell, 1969). Association of the increased incidence of infectious disease and accompanying higher mortality rates, particularly in infants and young children from countries where protein-calorie malnutrition exists, was a stimulus to nutritionists to investigate the role of dietary protein in the immune response. Much of the disease-resisting capacity of individuals lies in the gamma globulin fraction of plasma proteins. Synthesis of gamma globulin, like other tissue proteins, is regulated by the supply of available amino acids, which is, in turn, dependent on the quantity and quality of protein in the diet.

Numerous clinical and field studies (Scrimshaw et al., 1968; Béhar et al., 1958; Dubos and Schaedler, 1959; Hodges et al., 1962) have strongly associated dietary protein intake with resistance to infectious disease. Studies with animals have demonstrated specific relationships of quantity and quality of protein and effects of singular amino acids ingested on the immune response (Cannon, 1942; Guggenheim and Buechler, 1948; Kenney et al., 1965, 1968, 1970; Bhargava et al., 1969; Gershoff et al., 1968; Gill and Gershoff, 1967; Smith and Kenney, 1969).

Several controlled studies with animals have demonstrated roles for certain vitamins in the immune response. In some cases the vitamin

enhanced the natural resistance of a host to an infective organism (Seronde et al., 1956); in other cases, the vitamin influenced synthesis of antibody to a specific antigen (Axelrod, 1953; Axelrod and Trakatellis, 1964; Axelrod, 1971; Harmon et al., 1963a, 1963b).

Information on the role of mineral elements in the immune response is limited. From clinical and field observations on human populations, Scrimshaw (1968) reported a few instances in which a mineral deficiency was either antagonistic or synergistic to the existence of an infecting organism. Studies with experimental animals indicate that, as for the vitamins, certain mineral elements may influence natural resistance mechanisms (Pahomov, 1970a, 1970b; Pavlov, 1970; Hitchings et al., 1949, Newberne et al., 1968) or may affect the synthesis of antibody to a specific antigen (Bardare and Vaccari, 1967; Ermenkova and Ermenkov, 1967; Antonova et al., 1968; Nalder et al., 1972).

Malnutrition is likely to be a general syndrome, however, and may involve multiple nutrient deficiencies, any or all of which may either enhance or reduce the severity of concomitant infections. Consequently, the relationship between nutritional status and resistance to infectious disease is intricate and not easily elucidated. It seems logical to approach the problem through an investigation of recognized interrelationships in nutrient metabolism. The importance of pyridoxylidene-amino acids or Schiff's bases in reactions of protein metabolism has been well documented by studies with humans and animals. A number of studies in vitro have established the existence of metal chelates of Schiff's bases (Christensen and Riggs, 1956; Christensen and Collins,

1956; Christensen, 1957; Matsuo, 1957; Pal and Christensen, 1959; Davis et al., 1961). In addition to these observations, several reports have linked metals with protein metabolism in humans suffering with kwashiorkor and protein-calorie malnutrition (Caddell and Goddard, 1967; Caddell, 1967, 1969; Linder et al., 1963; Montgomery 1960, 1961) and in experimental animals (Bunce et al., 1963; Cox, 1960; Schwartz et al., 1969, 1970). Finally, dietary pyridoxine has affected metabolism of a metal, magnesium, in the rabbit (Aikawa, 1960) and in the rat (Korbitz, 1970a).

Proteins, vitamins and minerals have each influenced the immune response when studied separately, and the metabolism of these nutrients is interrelated. Therefore, a study of how these metabolic interactions affect the immune response might help to clarify the overall relationship between nutritional status and resistance to infectious disease.

The particular minerals and amino acid chosen for this study were manganese, magnesium and tryptophan. Manganese supplementation of rations stimulated antibody production in rabbits (Antonova et al., 1968). Magnesium has been experimentally linked with protein metabolism in several ways, including activation of enzymes critical to formation of adenosine triphosphate (ATP) needed for peptide bond formation and activation and transfer of amino acids to soluble ribonucleic acid (sRNA) in protein synthesis (Novelli, 1967); stabilization of the organization of deoxyribonucleicacid (DNA), RNA and ribosomes (Dove and Davidson, 1962; Venner and Zimmer, 1966; Tissieres et al. (1959); Rodgers, 1966;

Hamilton and Petermann, 1959; Edelman et al., 1960); contribution to binding of messenger RNA (mRNA) to the 70S ribosome (Gros et al., 1961; Brenner et al., 1961) and to the binding of sRNA to a site on the 50S subunit of the 70S ribosome (Cannon et al., 1963). In addition, magnesium apparently affected tissue growth in the thymus, which is involved with the establishment of immunologically competent cells (Bois et al., 1969).

Tryptophan intake influenced titers of antibody in experimental animals (Gershoff et al., 1968; Kenney et al., 1970). Furthermore, tryptophan apparently had a role in the maintenance of polysomal aggregates in protein synthesis (Hori et al., 1967; Sidransky et al., 1968; Munro, 1968). Changes in the metabolism of tryptophan were observed during the course of infectious disease (Rapoport et al., 1970; Rapoport and Beisel, 1971).

Pyridoxine is essential for the normal metabolism of tryptophan in many species and may have importance in the metabolism of magnesium as well (Aikawa, 1960; Korbitz, 1970a). In addition, it may affect nucleic acid metabolism (Trakatellis and Axelrod, 1965), mRNA biosynthesis and the aggregation of polysomes (Montjar et al., 1965), functions in which tryptophan and magnesium may also participate. Finally, the effect of vitamin B_6 deficiency on antibody formation has been well established (Axelrod, 1953; Axelrod and Trakatellis, 1964; Kumar and Axelrod, 1968).

The purposes of this study were 1) to investigate whether variations in dietary magnesium and manganese, either the level fed or the ratio of these to other key mineral elements fed, can alter the immune response of young male rats to sheep red blood cells and 2) to investigate

interrelationships in magnesium, manganese, tryptophan and vitamin B_6 metabolism in young male rats fed diets low in magnesium and the effects of these interrelationships on immune responses to sheep red blood cells. To evaluate the effects of the dietary variables on the immune response, serum agglutinins and hemolysins and total serum gamma globulin were estimated by titration and by optical density measurements. Splenic RNA and DNA concentrations were determined to assess capacity of the spleen for protein synthesis, since it is a site for gamma globulin and antibody production. Femur concentrations of magnesium and manganese were assessed to provide an index of the nutritional status of animals in relation to these minerals.

REVIEW OF LITERATURE

The complex nature of the relationship between the existing nutritional status and the ability of man or animals to respond to an antigenic stimulus is well recognized. The importance of protein concentration and quality and the need for certain vitamins, minerals and amino acids for the production of antibodies, a common measure of the immune response, have been demonstrated. Investigation of interrelationships in the metabolism of these nutrients may contribute to an understanding of how nutritional influences affect immunobiological reactions.

The objectives of this study were to describe actions and interactions of manganese, magnesium, tryptophan and vitamin B_6 in the immune response of young male rats sensitized to sheep red blood cells. Hence, this discussion will review the influences of these nutrients on general protein metabolism, on selected histological and biochemical parameters, on the immune response specifically, and on metabolic interactions among the 4 nutrients.

Magnesium

Magnesium has been recognized as an essential nutrient since 1932 when Kruse et al. (1932) described the symptoms of its deficiency in the rat. The most distinctive signs include peripheral vasodilation, skin lesions and hair loss, hyperirritability, muscle dysfunction, calcification of soft tissues, retarded growth, defective bones and teeth, and a bloody or brown-pigmented exudate from the nose and mouth. Although every symptom does not appear in all species, magnesium has been shown to be essential for man and other animals in addition to the rat. Characteristics of magnesium deficiency have been described in recent

review articles (Wacker and Parisi, 1968; Wacker and Vallee, 1964; Gitelman and Welt, 1969; Aikawa, 1971). This section will review the role of magnesium in protein synthesis and metabolism, its possible relation to the immune response and its significance in the growth of thymic tissue.

Association with protein synthesis

Magnesium is recognized as an essential factor in several mechanisms involved with protein synthesis. Protein synthesis is an energy-requiring process and therefore dependent upon oxidative phosphorylation. Membrane-bound ATP-ases are enzymes which are thought to promote coupling of phosphorylation with electron transport (Wacker, 1969). These enzymes require magnesium for activity. Therefore, magnesium can be regarded as a cofactor for oxidative phosphorylation and possibly for protein synthesis as well since the ATP formed in oxidative phosphorylation is the source of the energy required for peptide bond formation.

Magnesium helps to maintain biological macro-molecular structures through stabilizing the organization of DNA, RNA and ribosomes. DNA molecules contain the information required for synthesis of the proteins characteristic of a particular cell. Mutations reflect changes in the chemical structure of DNA and are transmitted to subsequent generations of cells. The importance of stabilizing this chemical structure is apparent. Dove and Davidson (1962) demonstrated the role of magnesium in maintaining the structural integrity of DNA. At low ionic strength (3×10^{-4}M), increasing the magnesium ion concentration of highly polymerized calf thymus and bacterial DNA solutions dramatically enhanced the resistance of DNA to thermal denaturation. It was proposed that the DNA helix was strengthened by stoichiometric binding of magnesium to the negatively-charged phosphate backbone of the native DNA molecule, thereby reducing

the electrostatic repulsion between the strands. Similar findings were reported by Venner and Zimmer (1966).

Magnesium may also influence the structure of RNA. Rodgers (1966) showed that magnesium functioned in maintaining the structure of the high-molecular weight (1.07×10^6) fraction of ribosomal RNA. At low ionic strength (0.01-0.04 with tris as the counter ion), removing magnesium from Escherichia coli ribosomal RNA with EDTA caused 2 boundaries to appear after ultracentrifugation. The first was an expanded or unfolded form of RNA resulting from loss of the magnesium associated nonspecifically with RNA through electrostatic forces; this form was not sensitive to EDTA. A more expanded form of RNA, which was sensitive to the EDTA concentration, was responsible for the second boundary and was thought to result from changes produced in the molecule by loss of the magnesium specifically associated with the fixed phosphates along the polynucleotide chain. The effect of EDTA was reversed if sodium was added immediately, but reversibility was lost if sodium was withheld for a time. This suggested changes in the tertiary structure of the molecule, since thermal denaturation studies showed no changes in secondary structure. Fresco et al. (1966) reported similar findings for some transfer RNA's.

Ribosomes are the sites for protein synthesis within the cell. Magnesium has an important influence on the degree of association of ribosomal components. Chao (1957), working with yeast ribonucleoprotein, demonstrated that the aggregation of smaller components into the larger ones required for protein synthesis was dependent upon an adequate supply of magnesium. Tissieres et al. (1959) found 4 ribonucleoproteins in extracts from Escherichia coli with sedimentation coefficients of 30S, 50S, 70S and 100S. At concentrations of 10^{-3}M Mg^{++}, the 70S peak predominated. Raising Mg^{++} to 10^{-2}M resulted in a preponderance of the 100S fraction. Lowering

Mg^{++} to 10^{-4}M resulted in the presence of only 30S and 50S fractions. The 70S particle, recognized as a complete ribosome, was found to be a product of the union of one 30S and one 50S fraction in the presence of adequate magnesium. Protein synthesis occurs on an aggregate of several of these 70S ribosomes connected by messenger RNA. The sedimentation coefficients of fractions obtained were slightly different, but the effects of magnesium concentration on aggregation of ribonucleoprotein particles were confirmed by Edelman et al. (1960) and Hamilton and Petermann (1959) working with ribosomal fractions from rabbit reticulocytes and rat liver, respectively.

In studies leading to the recognition of messenger RNA as the intermediate carrier of information from genes to ribosomes, Brenner et al. (1961) and Gros et al. (1961) extended the role of magnesium to include the binding of messenger RNA to the 70S ribosome. This binding was reversible and was dependent upon the concentration of magnesium.

Soluble RNA molecules, also known as transfer RNA, bind amino acids and transfer them to sites on the ribosomes where they are attached to the growing peptide chain. Cannon et al. (1963) used washed ribosomes from Escherichia coli to show that this soluble or transfer RNA was bound to the 50S subunit of the 70S ribosome at high concentrations (10^{-2}M) of magnesium. S-RNA was released from the ribosome by lowering the magnesium ion concentration. This binding was not influenced by temperature or inhibitors of protein synthesis and did not require enzymes or an energy source.

Certain enzymes involved in protein synthesis are activated by magnesium. The first step of protein synthesis involves activation of the amino acid; the carboxyl group of the amino acid forms an anhydride with the phosphate of AMP. The second step involves transfer of the amino acyl compound to its specific tRNA. Amino acyl synthetase catalyzes both steps

and requires magnesium for its activity (Novelli, 1967). DNA-dependent RNA polymerase catalyzes the formation of messenger RNA and also requires magnesium (White et al., 1968).

Salas et al. (1967) and Gordon and Lipmann (1967) showed that in the final step of protein synthesis the interaction among ribosomes, messenger RNA and the transfer RNA carrying its amino acid required magnesium.

The information on effects of magnesium on macromolecules involved in protein synthesis from the preceding studies was derived from work done in cell-free artificially manipulated media. Zieve et al. (1972) reported on protein and nucleic acid synthesis in tissues from rats fed magnesium-deficient diets. They found that splenic DNA synthesis was tripled while protein synthesis was halved in comparison to control rats. RNA synthesis in the spleen was not changed. These parameters were not significantly affected in the liver, kidney or thymus. Studies in vitro of protein synthesis in spleen extracts showed that ribosomal structure and function was normal. This finding might seem to contradict the preceding studies but still would not rule out an effect of magnesium on ribosomal structure or function, because the intracellular magnesium of the spleen may not have been lowered enough to affect these parameters. Also, the number of normally functioning ribosomes may be reduced in magnesium deficiency. There was no data presented to indicate whether a comparative ribosomal profile was prepared but since rate of protein synthesis is generally proportional to number of ribosomes present, this might explain the reduced rate of protein synthesis observed. Since supernatant fractions from magnesium-deficient animals were much less active in protein synthesis than those from control rats, it is likely

that the effect of magnesium was exerted at the level of enzymic amino acid activation, transfer RNA integrity, or peptide chain formation.

Schwartz et al. (1970) found a significant reduction in serum albumin synthesis but not in total soluble protein synthesis in livers of magnesium-deficient rats. This decrease in serum albumin synthesis, as an indication of reduced levels of protein synthesis in magnesium deficiency, is in agreement with the findings of Zieve et al. (1972) on splenic protein synthesis reported above. Zieve's group also failed to demonstrate an effect of magnesium deficiency on liver protein synthesis, but they did not look at albumin synthesis. Schwartz et al. (1970) also looked at cell-free systems prepared from the livers of magnesium-deficient rats and pair-fed controls to locate any changes in the protein synthetic capacity of subcellular fractions. In agreement with Zieve et al. (1972), they found no structural or functional changes in the polysomes, but did find a change in protein-synthetic capacity in the supernatant fraction.

From this review, it is apparent that magnesium has 3 general functions related to protein synthesis: 1) activation of enzymes catalyzing reactions in the essential biological processes of protein synthesis and oxidative phosphorylation, 2) maintenance of the structure of the individual macromolecules involved and 3) stabilization of the interaction of these macromolecules so that synthesis of the polypeptide chain on the ribosomal complex can proceed. The importance of maintaining an optimum intracellular concentration of magnesium in vivo has been supported by observations of disturbed protein synthesis in experimental

animals deficient in magnesium.

Experimental studies with humans give little basis on which to generalize whether dietary protein influences magnesium utilization, because of differences in age and sex of subjects, level and source of protein, composition of basal diets, and length of experimental periods. This review will be limited, therefore, to animal studies which allow inclusion of more stringent controls over experimental conditions.

Several studies have reported an interdependence of magnesium and dietary protein. Menaker (1954), using weight gain as a measure of protein synthesis, reported that female rats, reduced 20-25% below initial weights of 250 gm and fed magnesium-deficient mineral supplements in their rations, gained twice as much weight on a diet containing 7% protein as on a diet containing 14% protein.

In contrast, Vitale et al. (1959) found that rats fed a 10% casein, 20% fat diet required the inclusion of 4 to 8 times as much magnesium to prevent signs of deficiency as rats fed a 20% casein, 5% fat diet. Since magnesium is essential for fat metabolism also, the difference in fat intake may have obscured the effect of protein on magnesium utilization.

Cox (1960) also failed to support the observation of Menaker (1954) that increased casein in diets of magnesium-deficient rats intensified the deficiency symptoms. She found that animals fed 28% casein without added magnesium were better off than animals receiving 14% casein without magnesium. They had greater nitrogen retention, more hepatic nitrogen and higher serum albumin levels. Differences in the findings of these 2 studies might be explained on the basis of different casein concentrations

fed. Also, Menaker's (1954) diets were fed for only 10 days, as compared to 84, 42, and 28 days for most groups in Cox's experiment (1960). Possibly, during the longer feeding period, metabolic adaptations to magnesium deficiency occurred so that animals benefited from more dietary protein. However, this possibility was not borne out by the findings of Schwartz et al. (1969) and Bunce et al. (1963) in experiments lasting 5 or 6 weeks, respectively. Finally, utilization of protein and the consequent metabolic interactions of protein and magnesium may have been quite different in the female rats repleting body weight in Menaker's study and in the growing, male rats of Cox's study.

Bunce et al. (1963) observed depressed weight gain with increasing dietary protein-to-magnesium ratios in chicks and in male weanling rats. Magnesium-deficiency symptoms appeared in rats fed diets containing 200 ppm magnesium on 36% protein diets, a level of magnesium normally considered adequate. In addition, Bunce et al. (1963) found abnormally high urinary excretions of leucine, isoleucine and valine, along with increased urinary magnesium losses and decreased levels of magnesium in livers from deficient animals. They suggested that there was a greater turnover of magnesium in animals on high protein intakes.

Schwartz et al. (1969) confirmed the observations of Menaker (1954) and Bunce et al. (1963) that feeding high protein diets with marginal levels of magnesium hastened the rate of magnesium depletion and appearance of magnesium deficiency signs in weanling rats. They found that dietary magnesium levels of 0.01, 0.05, and 0.10% caused progressive increases in weight gain, total plasma protein, and in dietary nitrogen

retention in rats fed 36% protein from casein thus confirming the concept that magnesium requirement is increased when protein intake increases. In contrast to Bunce et al. (1963), these investigators found that less urinary magnesium was lost with 36% protein diets than with 12% protein diets. This difference was explained on the basis of differences in experimental conditions - strain of rats used, greater initial weights of animals, shorter experimental period, time periods for which magnesium balances were determined, and method used for magnesium analysis.

In summary, information from several animal studies indicates that feeding high levels of protein to growing animals and to animals repleting body weight often caused a greater requirement for magnesium, since effects of magnesium depletion were generally augmented with increases in dietary protein.

Relation to the immune response

Normally leukocytes, through phagocytic action, provide a rapid and potent defense for the body against infectious agents which may invade it. In leukemia or uncontrolled leukocytosis these cells are usually nonfunctional and do not provide the usual protection associated with white blood cells. Battifora et al. (1968) studied chronic magnesium deficiency in two-month-old, male rats over a period of several months and found that rats fed about 5 mg. of magnesium per 100 gm diet developed neutrophilic leukocytosis. This condition was usually reversed by feeding adequate magnesium, but 10% of the rats developed a more severe, nonreversible leukocytosis along with other characteristics of chronic,

myelogenous leukemia. Bois et al. (1969) produced lymphoid leukemia in 6-7 week old male rats by feeding a magnesium-deficient diet. These investigators did not evaluate the immune response of their magnesium-deficient animals, but the implication for further research seems obvious.

McCreary et al. (1966) observed that the characteristics of acute magnesium deficiency in the rat, such as hyperemia of the skin, leukocytosis, eosinophilia, mast cell degranulation, and increased urinary excretion of histamine, resembled the conditions seen in allergic reactions and were, therefore, suggestive of an altered immunological state. They tested the response of magnesium-deprived rats to guinea pig spinal cord homogenate, a stimulus for the production of experimental allergic encephalomyelitis (EAE), and found that magnesium deficiency was protective against the development of encephalomyelitis. Seventy percent of control animals and 18% of magnesium-deficient rats developed histological evidence of EAE. The degree of EAE in magnesium-deficient rats was inversely proportional to the severity of magnesium deficiency signs. They concluded from these observations that a hyperimmune state did not exist in magnesium deficiency, since tolerance to the EAE allergenic stimulus was increased. They could give no explanation for this increased tolerance. It is interesting to speculate, however, that this tolerance might have been induced as a result of impaired ability to produce antibodies to the guinea pig spinal cord homogenate, since the first stage in anaphylactic sensitization is the production of antibody capable of binding to tissue cells, especially mast cells (Stewart, 1968). McCreary et al. (1966), in conjunction with the EAE experiment, also

tested the ability of their magnesium-deficient rats to produce antibody against pertussis vaccine, but found no differences between control and deficient groups. From their discussion, it appeared that antipertussis agglutination titers were determined approximately 18 days after injection of the vaccine; effects of magnesium depletion on antibody production could have been masked if peak antibody production occurred earlier than 18 days. Also, the animals had been on the depletion diet for only three days when the antigen was injected, so their ability to form antibody to this particular antigen may not yet have been impaired.

The gamma globulin fraction of plasma proteins contains much of the body's disease-resisting capacity. Cox (1960) reported that the percent of gamma globulin decreased in serum of rats fed a low-magnesium diet for 84 days.

Immunoglobulin G, also known as 7S antibody, is an antigenically distinct globulin which accounts for about 70% of total immunoglobulin. Alcock and Shils (1973) reported low levels of serum immunoglobulin G (Ig G) in magnesium-deficient, adult, male rats. Magnesium-repleted animals had a two-fold rise in IgG as compared to deficient animals after 10 and 28 days of repletion. They concluded that magnesium is required for the maintenance of normal levels of IgG in the rat.

Complement is a term applied to a group of serum globulins which can combine with antigen-antibody complexes and which mediate the lysis of cells sensitized by antibody. Combination of complement with antigen-antibody complexes is referred to as complement fixation. McCreary *et al.* (1966) suggested that even if normal amounts of antibody are produced in

magnesium deficiency, its activity may be altered by low tissue-magnesium levels, since magnesium is required for complement fixation in vitro (Levine et al., 1953).

In summary, it has been reported that magnesium has possible effects on the immune response at the level of the control of leukocyte formation, the control of immunoprotein formation (i.e., total gamma globulin or specific fractions of gamma globulin such as immunoglobulin G), or at the level of the complement fixation reaction.

Significance in thymic tissue growth

The thymus plays a major role in the establishment of immunological function in the young animal through production of a population of immunologically competent lymphocytes. Stewart (1968) has suggested 3 main ways in which the thymus may exercise immunological control:

1. "It might be responsible for the initial seeding, during immunological development, of lymphoid tissues with lymphocyte precursors.
2. Lymphocyte precursor or stem cells may be transformed into cells with immunological capacity by migration through the thymus, where they undergo transformation through the action of a hormone secreted by the thymic reticular cells.
3. Lymphocyte precursor or stem cells may be transformed by thymic hormone in situ in lymphoid tissues, without the necessity for migration through the thymus." (p. 124).

Bois et al. (1969) reported the development of thymic tumors in 20.6% of survivors from 402 young, male rats maintained on magnesium-deficient

diets for 8 weeks or more. Examination of tissue revealed that neoplastic cells completely replaced the normal structure of the thymus. Among the changes noted in the reticular cells and lymphocytes of deficient animals were more numerous, small, free ribosomes in the cytoplasm and a difference in the adhesion of cells to one another, properties which have been directly related to magnesium concentration in vitro (Tissieres et al., 1959; Edelman et al., 1960; Hamilton and Petermann, 1959). In view of the recognized importance of the thymus in the immune response, the changes induced in it by magnesium deficiency could have a profound effect on resistance mechanisms, particularly in the young animal.

Manganese

Manganese is essential in the diets of several species, including mice, rats, rabbits, cows, pigs, guinea pigs, and chickens. It supports normal growth and reproduction in animals, having a particularly important effect on skeletal growth and development through its action in mucopolysaccharide synthesis. Congenital ataxia has also been commonly observed in manganese-deficient animals and has been linked to a genetic mutation which causes a higher requirement for manganese in mice and rats (Cotzias, 1962; Erway et al., 1970; Hurley, 1968). This section will discuss the role of manganese in amino acid metabolism and its relation to the immune response.

Relation to amino acid metabolism

The ability of manganese to form chelates with Schiff's bases or pyridoxylidene amino acids has been established (Christensen and Collins,

1956; Christensen, 1957; Matsuo, 1957; Davis et al., 1961). The study of Pal and Christensen (1959) demonstrating the stimulatory effect of certain amino acids and pyridoxal on manganese uptake by Erhlich ascites tumor cells has important biological implications. The basic amino acids lysine and diaminobutyrate were more effective when administered alone, suggesting some specificity for amino acid structure as well as for the metal concerned, since uptakes of zinc, iron, or copper were unaffected. Addition of glycine, which had no effect on manganese uptake when administered alone, of lysine or of diaminobutyrate along with pyridoxal caused the greatest stimulation of manganese uptake. Pal and Christensen postulated that either manganese or pyridoxal or both facilitated amino acid transport.

In addition to its participation in amino acid metabolism through its chelating properties, manganese is an activator of arginase, the enzyme which catalyzes the irreversible hydrolysis of arginine to ornithine and urea (White et al., 1968). Urea in many species is the primary mode of excretion for excess nitrogen.

Relation to the immune response

Several studies have indicated a role for manganese in the immune response of horses, goats, mice, hens, and rabbits. In a review article, Cotzias (1962) reported from a translation of early papers (1923 and 1925) by Walbum and co-workers in Germany showing a stimulatory effect of injected manganese and cobalt chlorides on diphtheria antibody titers of goats and horses. Manganese chloride was more effective than cobalt chloride when injected but not when fed. Manganese concentration of

organs from these animals increased in proportion to the amount of salt injected, and liver manganese was directly related to antibody titer.

Hitchings and Falco (1946) found that mice receiving a purified diet survived the equivalent of 10^5 lethal doses of Type I pneumococcus injected into animals fed a commercial laboratory diet. This observation caused them to attempt to isolate from the commercial diet the factor which increased the susceptibility of the mice to pneumococcal infection. They subsequently identified the chief component of the factor as manganese and showed that the susceptibility of mice to infection steadily increased with increasing dietary manganese content to levels as high as 0.6 mg manganese per gram of diet (Hitchings et al., 1949). They could antagonize the manganous effect in some cases by excesses of dietary cobalt. No explanation for this effect of manganese was offered.

Ermenkova and Ermenkov (1967) found that a mixture of manganese, cobalt, and iodine given as a supplement to the diet of laying hens stimulated antibody formation to red blood cells and maintenance of the titers generated. They found no change in total serum protein or in any of its major fractions. Again, no explanation for the action of the trace-element mixture was offered.

Antonova et al. (1968) investigated the influence of small amounts of trace elements on the immune response of rabbits. Copper, manganese, and titanium were fed at a level equivalent to 3 times the normal dietary level. After 3 months of feeding, the nonspecific agglutinins in the blood were determined and phagocytic activity of the leukocytes was determined as an index of natural immunity. After 4 months of feeding,

the rabbits were immunized 6 times at 5-day intervals with a preparation of Escherichia coli. Agglutination reactions and phagocytic activity of leukocytes were tested on the seventh or eighth day after final immunization. Copper, manganese, and titanium supplementation stimulated the immunological reactions as assessed by each of the parameters evaluated for both natural immunity and for the artificially-produced immunity. No mechanism was proposed to explain the effect of the trace elements.

To summarize, administration of manganese in excess of requirements, by injection or in the diet, affected the immunological reactions of a number of animals. For horses, goats, hens and rabbits the effect was to enhance antibody production and other resistance factors. For mice, the effect was to increase mortality rates. None of the studies postulated mechanisms for the action of manganese, nor did they report biochemical parameters or assessments of histological changes. Since species and methods of administering manganese were also widely different, it is impossible to explain or generalize the findings except to say that manipulating manganese concentration within a living organism appears to influence its resistance mechanisms.

Tryptophan

Tryptophan belongs to the class of amino acids termed "essential" because it cannot be synthesized by the animal organism at a rate sufficient to meet its metabolic requirements; it therefore must be supplied in the diet. Both the essential and nonessential amino acids must be available simultaneously and in sufficient quantities to support

efficient protein synthesis. When the metabolic pool of an amino acid is depleted the tissues cannot make proteins which contain that particular amino acid, at least not at normal rates of synthesis. Tryptophan is a common, limiting amino acid in food sources. This section will review the participation of tryptophan in the basic control of protein synthesis and its effects on the immune response.

Participation in control of protein synthesis

Several papers from the literature have implicated tryptophan as a controlling factor in protein synthesis. Hori et al. (1967) found that of all the amino acids required for optimum hemoglobin synthesis in isolated rabbit reticulocytes, only a deficiency of tryptophan in the incubation medium resulted in polyribosome disaggregation. They proposed that the effect of tryptophan was a result of its unique position near the amino-terminal ends of both the alpha and beta chains of rabbit hemoglobin in position 14 and positions 15 and 37, respectively. Growth of a peptide chain begins at the amino-terminal end, so tryptophan deficiency would retard translation of messenger RNA before a sufficient number of ribosomes were attached to the messenger RNA to maintain a steady state. Valine, an amino acid more essential than tryptophan for rabbit-hemoglobin synthesis, occupies the amino-terminal position of both the alpha and beta chains of rabbit hemoglobin, as well as several other positions within the chain. Valine deficiency failed to produce polyribosomal disaggregation, possibly because fewer chains could be initiated and because growth of previously initiated chains was interrupted

after a sufficient number of ribosomes had been accumulated on messenger RNA to maintain the steady state. Double deficiencies of tryptophan with amino acids more essential for hemoglobin synthesis than itself (histidine, valine, leucine, phenylalanine) resulted in no polysomal disaggregation. Tryptophan deficiency coupled with deficiency of amino acids less essential for hemoglobin synthesis than itself (lysine, serine, tyrosine) resulted in polysomal disaggregation. Presumably, tryptophan is the limiting amino acid for hemoglobin synthesis in the latter case but not in the former, in which enough tryptophan to support limited synthesis could come from breakdown of other cell protein.

Swan et al. (1971) investigated factors affecting ribosomal aggregation in the isolated perfused rat liver and found that omission of either tryptophan or methionine from the perfusate resulted in disaggregation of polysomes. Methionine deficiency seemed more detrimental than tryptophan deficiency because polysomes did not reaggregate when it was added to the medium. No explanation was proposed for either effect, but, in the discussion following the paper, Korner mentioned that methionine may act as the N-terminal amino acid during protein synthesis in mammalian cells. If this is true, it would follow that ribosomal aggregation may not occur because the peptide chain could not be initiated.

Sidransky et al. (1968) found that tryptophan deficiency resulted in lighter aggregates of hepatic ribosomes in tube-fed mice. Hepatic protein synthesis as measured by incorporation of ^{14}C-leucine or ^{14}C-phenylalanine in vitro or ^{14}C-leucine in vivo was also depressed in mice receiving the tryptophan-deficient mixture. It was proposed that

the effect of tryptophan was at the translational level as a limiting amino acid rather than at the transcriptional level, since the polyribosomes continued to respond to tryptophan even when RNA synthesis was strongly inhibited by actinomycin D. Later findings of Sidransky et al. (1971) noted a shift toward heavier polyribosomes and increased in vitro hepatic protein synthesis in rats to which tryptophan had been administered. They also looked at protein synthesis in liver tissue and in hepatoma tissue transplanted to the same rat and found that protein synthesis increased in liver but not hepatoma tissue when tryptophan was administered. This shows that specific proteins may be more sensitive to tryptophan control than others.

Baliga et al. (1968) and Munro (1968) have proposed that the unique effect of tryptophan in causing changes in the polysomal profile of the whole animal cannot be attributed to a special role of tryptophan in the mechanism of protein synthesis. They were able to produce polysomal disaggregation in a cell-free system when any one of a number of amino acids were withheld. On this basis they proposed that the unique sensitivity of polysome pattern to tryptophan could be related to the fact that trptophan is the least-abundant amino acid both in the free amino acid pool and in the proteins formed from the pool in intact animals. Hori et al. (1967), Swan et al. (1971), and Sidransky et al. (1968, 1971) worked with intact animals, tissues or cells and probably had not succeeded in reducing the intracellular concentration of amino acids other than tryptophan to the point that they became rate-limiting at the translational level of protein synthesis with the exception of methionine

in Swan's study (1971).

Another possible level for tryptophan participation in control of protein synthesis is through an effect on DNA-dependent RNA polymerase activity. DNA-dependent RNA polymerase is an enzyme which, when activated by magnesium, catalyzes the formation of messenger (White et al., 1968) and ribosomal RNA's (Henderson, 1970). Henderson (1970) fed a tryptophan-free amino acid mixture and studied nuclear magnesium-activated DNA-dependent RNA polymerase of livers from young, male albino rats, starved for 18 hours prior to feeding and killed 15-120 minutes later. There was a rapid increase in free tryptophan concentration in both plasma and liver after feeding a complete amino acid mixture. Feeding the tryptophan-deficient mixture caused no change in the plasma tryptophan concentration; liver tryptophan concentration increased after about 45 minutes. There was a rapid, essentially linear response of the polymerase over the first hour after feeding the complete mixture; activity of the enzyme almost doubled during this period. Response of the enzyme to feeding the tryptophan-deficient mixture was much weaker and was delayed about 45 minutes post-feeding. Liver tryptophan concentration and enzyme activity were well-correlated in all groups. Puromycin administration affected the enzyme differently according to diet, abolishing the 15 minute enzyme response in livers from animals fed the complete amino acid mixture. The optimum magnesium and hydrogen ion concentrations for enzyme activity also seemed to differ between groups. RNA polymerase and orotic acid incorporation by liver nuclei were affected similarly by the amino acid mixtures. From these differences, it was concluded that a new relatively

short-lived RNA polymerase was synthesized in rats fed the complete mixture. It was thought that the synthesis of this new polymerase in response to increased amino acid supply may be a means of regulating RNA synthesis by amino acid concentration. The effect of tryptophan concentration was not believed to be specific, but to reflect the general amino acid concentration required to support hepatic protein and polymerase synthesis.

In summary, tryptophan may have an effect on the control of protein synthesis at the ribosomal level or at the level of induction of enzyme formation. It is not completely clear whether this is a specific effect or whether it is a result of being the most-limiting amino acid in the metabolic pool, although there is perhaps more evidence in support of the latter theory. More work to characterize specific rather than total protein synthesis may help to clarify the role of tryptophan in control mechanisms.

Effects of dietary tryptophan on the immune response

A number of studies have shown that the tryptophan concentration of the diet of an animal influences its immune response. Axelrod (1953) reported the effects of a niacin-tryptophan deficiency on antibody production in rats fed 9% casein-40% corn grits diets with no niacin supplement and immunized with human red blood cells. Lowered agglutinin activity was noted in niacin-tryptophan deficient animals. These could be restored to high-normal control values by supplementing diets with 0.4% tryptophan but not with 0.15 mg niacin per day. Titers in pair-fed

control animals were also higher, showing that tryptophan deficiency was the controlling influence on antibody formation and not food intake.

Kenney et al. (1970) reported that tryptophan was functional in maintaining immunoglobulin concentrations in young growing male rats and protein-depleted adult male rats. Isonitrogenous diets containing 9-10% protein from soy alpha-protein, wheat gluten, corn or a mixture of corn and casein, with or without supplements of the limiting amino acids were fed. Antibody formation to sheep red blood cells was measured between 4 and 21 days after immunization. Tryptophan supplementation of corn or corn-casein diets increased hemolysin titers of adults and agglutinin titers of young rats 6 days after immunization. Levels of tryptophan in excess of the growth requirement did not improve agglutinin titers but hemolysin titers increased linearly with level of tryptophan fed in the range of 0-2.25 mg per gram diet. The concentration of 7S antibody was elevated on days 7, 14, and 21 in the growing rats fed tryptophan; there was no effect of the amino acid on 19S titers 7, 14, and 21 days after injection.

Gershoff et al. (1968) studied the effects of feeding tryptophan-deficient (0.04%) diets on the immune response of weanling rats fed the diets 5 days prior to and 9 days after immunization with sheep red blood cells. Tryptophan-deficient animals had lower hemagglutination titers than control animals.

From these studies it appears that tryptophan affects the ability of animals to form antibodies to a specific stimulus. The specific effects may differ with such factors as age, time elapsed after

immunization, and specific immunoglobulin measured. The general depression in the immune response caused by tryptophan deficiency is apparent, however.

Vitamin B_6

The term vitamin B_6 is used as a general designation of 3 compounds which function as members of the vitamin B complex. The 3 forms are referred to specifically as pyridoxine, pyridoxal and pyridoxamine, depending on the chemical composition of the isolate. A phosphorylated derivative of pyridoxine known as pyridoxal phosphate is active in many of the biological reactions in which vitamin B_6 participates. The extensive participation of vitamin B_6 in reactions of protein and amino acid metabolism is well-known. Promoting increased intestinal absorption of amino acids, aiding in amino acid concentration by cells, and participation in enzymically catalyzed syntheses and interconversions of amino acids within the organism are among its functions. A detailed discussion of these many roles is beyond the scope of this review. A number of reviews have covered this subject adequately. Consequently, this one will be limited to consideration of the role of vitamin B_6 as it is related to the basic control of protein synthesis and to the immune response.

Relation to protein synthesis and metabolism and to the immune response

Numerous investigators have documented the nutritional significance of vitamin B_6 in the immune response of organisms to a variety of antigenic stimulants (Axelrod and Trakatellis, 1964). Axelrod (1953) found

hemagglutinin titers to be severely reduced in young, albino rats fed a pyridoxine-deficient diet and immunized with human erythrocytes. Inanition controls were employed and the high hemagglutinin titers from these animals indicated the depressed antibody production was a function of B_6 deficiency and not of decreased food intake. Similar results were obtained by Gershoff et al. (1968). They found that supplementation of diets with glycine or serine could reverse the inhibition of antibody formation caused by B_6 deficiency. Glycine and serine are sources of one-carbon units needed for syntheses of purine and pyrimidine bases used in production of nucleic acids.

Subsequent work by Axelrod and his co-workers has employed a coordinated approach to understanding the precise role of vitamin B_6 in antibody synthesis. Their first hypothesis was that since antibodies are proteins, the inhibitory effect of pyridoxine deficiency on antibody synthesis could be a reflection of the requirement for B_6 in the general process of protein biosynthesis. To test this hypothesis, they looked at the effects of pyridoxine deficiency on the incorporation of L-valine-1-C^{14} into liver, spleen and serum proteins of rats and found consistently decreased incorporation rates in deficient animals. The effect was less, however, than the inhibitory effect on antibody synthesis noted in prior studies, but the possibility that pyridoxine functions similarly in antibody synthesis and in general protein biosynthesis was not ruled out (Trakatellis and Axelrod, 1964).

A further study (Trakatellis and Axelrod, 1965) used labeled precursors (serine, adenine, thymidine and deoxyuridine) to show that

nucleic acid synthesis was diminished in liver and spleen. Spleens from pyridoxine-deficient rats contained fewer cells and a lesser concentration of DNA than those from control animals. From these results, it was postulated that the decreased capacity for DNA biosynthesis in deficient animals, along with the subsequent deleterious effect on cell multiplication, could explain the adverse effects of pyridoxine deficiency on immune responses. An additional conjecture was that the decreased incorporation of valine into tissue protein from the previous study (Trakatellis and Axelrod, 1964) may have been a result of insufficient production of the necessary messenger RNA.

The 1965 study had linked the action of pyridoxine in protein biosynthesis to its role in nucleic acid metabolism. Montjar et al. (1965) investigated this relationship further by assessing the effect of B_6 deficiency on the polysome content of liver and spleen, on the ability of these tissues to incorporate amino acids in vitro, and on the rate of ribosomal and messenger RNA synthesis. Inanition controls were used for comparative purposes. The B_6-deficient rat had fewer polysomes per unit weight of liver and spleen in each category of aggregate polysome size. Purified ribosomes from rat liver and spleen in a cell-free system were used to study tissue incorporation of amino acids. Cell-free systems of liver from B_6-deficient rats incorporated labeled leucine and labeled valine less readily. Labeled leucine was also incorporated less readily by cell-free spleen preparations from B_6-deficient animals, amounting to 60-75% of the values obtained from the inanition controls. There was also a decreased incorporation of labeled orotic acid into the 29S and 18S

components of ribosomal RNA and into messenger RNA. The decreased number of polysomes per unit weight of liver and spleen and the consequent decreased capacity of these tissues to incorporate labeled amino acids *in vitro* probably resulted from the inhibition of ribosomal and messenger RNA synthesis in B_6 deficiency.

These studies had shown the deleterious effects of B_6 deficiency on total cellular proliferation and on the synthetic capacity of the cell. Kumar and Axelrod (1968) attempted to demonstrate whether B_6 deficiency affects antibody-forming cells in the same way as total cells. The Jerne agar-plaque technique was used to determine the number of antibody-forming cells (AFC) in the spleen. The number of AFC was markedly reduced in spleens from B_6-deficient animals. The effect did not seem to cause irreversible degeneration of immunologically competent cells, however, because pyridoxine therapy (5 mg/day injected intraperitoneally) for 3 days prior to immunization and continued for 4 days until autopsy caused an increase in AFC to control levels. Decreased numbers of AFC were accompanied by markedly depressed serum hemolysin values.

From the results of these studies Montjar *et al.* (1965) have proposed a unified concept of the role of B_6 in antibody formation:

> "It is known that administration of an antigen stimulates extensive multiplication of host cells in certain organs concerned with immune responses, e.g., spleen and lymph nodes. Although the mechanism by which the antigen excites this proliferation is not clear at the present, there is no doubt that accelerated synthesis of DNA is required at this step in the immune process. By inhibiting DNA synthesis, a deficiency of pyridoxine could prevent the required cellular proliferation and, consequently, inhibit antibody production. Our previous observation that pyridoxine-deficient rats possessed fewer cells per unit weight of splenic tissue conforms with this concept. Furthermore, an accelerated production of specific messenger RNA possessing the information necessary for

> synthesis of antibody protein would be expected after antigenic stimulation under normal conditions. In pyridoxine deficiency, therefore, the impairment of synthesis of m-RNA as well as ribosomal RNA, could also effectively reduce antibody synthesis. Thus, the deleterious effects of a pyridoxine deficiency upon immunological phenomena may be exerted at the site of cellular proliferation as well as on the synthetic capacity of the cell."

This hypothesis was advanced prior to the study of Kumar and Axelrod (1968) using immunized rats, but their results were in accord with the proposal.

Biochemical Interrelationships of Mg, Mn, Tryptophan and B_6 Metabolism

Magnesium-manganese

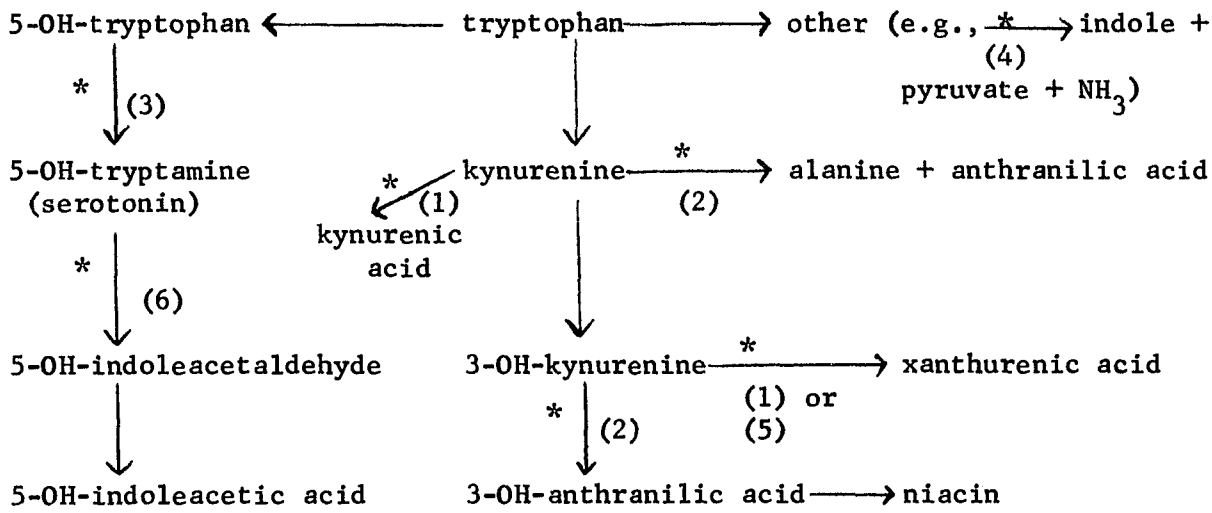
The biological essentiality of the metals magnesium and manganese have been demonstrated. Information gained from work with tissue slices, enzymes, and unicellular organisms, models presumed to represent body physiological systems, indicated that the metal requirement of the systems studied could be satisfied by either magnesium or manganese. Cotzias (1960, 1961) looked at the large differences in body concentrations of manganese and magnesium, the differences in nutritional requirements, modes of excretion and manifestations of deficiency and toxicity *in vivo* and thought the evidence strong enough to justify an experiment to determine the specificity of metabolic pathways for the 2 metals in living systems. He used an isotopic technique which permitted the direct assay of the displacement of one metal by another in the body. An isotope of manganese chloride was injected parenterally into mice and body radiation and initial rates of excretion were ascertained. Injections of magnesium, members of the first transition group of elements, and of members of

period VII, in highly significant amounts relative to estimated total body contents of each, were given to mice. Changes in the relative concentrations of isotopic manganese chloride and other elements were determined by changes in radioactivity emitted by the whole body. Only manganese was effective in displacing radiomanganese within the body, indicating that the metabolic pathway of manganese through the body is specific. Specificity appeared to be a function of a beta-1-globulin of blood plasma which binds manganese in the trivalent state. Magnesium exists in the divalent state *in vivo*. The property of physiological systems in living organisms for differentiation between manganese and magnesium may be a result of the oxidation state of manganese. Foradori *et al*. (1967) investigated the discrimination between magnesium and manganese by serum proteins and found that manganese is selectively bound by a beta 1-globulin, confirming the concept that "plasma proteins contribute to biological specificity by discriminating between a trace metal and a macronutrient."

Tryptophan-vitamin B_6

Metabolism of tryptophan, a complex heterocyclic amino acid, gives rise to several biologically-active compounds. Serotonin, a strong vasoconstricting substance, and niacin, a member of the vitamin B complex and an important constituent of the coenzymes of electron transport NAD and NADP, are perhaps the most important in terms of biological activity. Pyridoxal phosphate is essential for the activity of several enzymes participating in tryptophan metabolism. Sauberlich (1968) summarized the

major pathways of tryptophan metabolism in the following scheme:



*pyridoxal phosphate required

(1) kynurenine transaminase (2) kynureninase (3) aromatic L-amino acid decarboxylase (4) tryptophanase (5) "hydroxykynurenine transaminase" (6) monoamine oxidase

These are the specific enzymes which require pyridoxal phosphate. Comparison of the urinary excretory products in vitamin B_6 deficiency or when large amounts of tryptophan were fed with urine from control animals helped to identify some of the metabolic pathways in which vitamin B_6 participated because of the appearance of abnormal amounts of the metabolites of tryptophan, xanthurenic acid, kynurenine, and 3-hydroxykynurenine.

Tryptophan-magnesium

Korbitz (1970b) reported on tryptophan metabolism in the magnesium-deficient rat. Rats were fed magnesium-deficient diets and then injected intraperitoneally with tryptophan. It was concluded that magnesium

deficiency alone did not significantly affect tryptophan metabolism, probably because other metals such as manganese or zinc could also activate pyridoxal enzyme systems. Tryptophan was administered by intraperitoneal injection, so it was possible that it was not metabolized in the same manner as dietary tryptophan.

Magnesium-vitamin B_6

Aikawa (1960) studied the effects of pyridoxine and its metabolic antagonist desoxypyridoxine on magnesium metabolism in the rabbit. He conducted external balance studies for magnesium and isotopic determinations of exchangeable magnesium in the bodies of adult male rabbits before and after injections of 2 levels of pyridoxine or desoxypyridoxine. The higher level of pyridoxine resulted in an increased uptake of magnesium by appendix and heart. Animals given desoxypyridoxine had a significantly decreased uptake of magnesium in kidney, lung, and bone. Either an excess or a deficiency of pyridoxine altered magnesium uptake. The elevation of serum magnesium along with an unaltered urinary excretion of magnesium possibly indicated that pyridoxine affected the distribution of magnesium between intra- and extra-cellular compartments.

Korbitz (1970a) apparently intensified magnesium deficiency in rats by adding 50 mg of pyridoxine hydrochloride per 100 grams diet, equivalent to 200 times the normal dietary level. Mortality rates of magnesium-deficient rats increased when the high level of pyridoxine was fed. There was no change in the mortality rate of another group of rats fed 200 times the normal amount of thiamine. Pyridoxine level caused no

consistent difference in serum magnesium levels of magnesium-deficient rats. It was proposed that a high dietary pyridoxine level increased the demand for all activator cations (i.e., Mg^{++}, Mn^{++}, Zn^{++}) for pyridoxal phosphate-linked enzyme systems. When one cation was deficient the pyridoxine load augmented the deficiency state, the effects of which were manifested by the increased mortality rate in this study.

It is not possible to generalize on interrelationships of magnesium and vitamin B_6 from the 2 studies reported in the literature because of differences in species tested, mode of administration of B_6, dosage of B_6 given, length of experimental period. It does appear that pyridoxine and magnesium metabolism are related, however.

Tryptophan-magnesium-vitamin B_6

Gershoff and Andrus (1961) studied renal calculi formation in pyridoxine-deficient rats and found elevated levels of oxalic acid and xanthurenic acid and depressed levels of citrate in urine. When high levels of dietary magnesium were given the citrate levels returned to normal and xanthurenic acid levels were significantly decreased. The effect of magnesium on xanthurenic acid level was not clear, but pyridoxine deficiency may have in some way sensitized magnesium-activated pyridoxal phosphate-linked enzymes involved in tryptophan metabolism to the high levels of magnesium fed so that limited amounts of coenzyme were bound firmly and utilized more efficiently.

Summary of interrelationships reviewed

Studies reviewed in this section reveal that in the living animal magnesium and manganese are probably not functionally interchangeable because magnesium exists as a divalent cation *in vivo* while manganese exists in the trivalent state and is found specifically by a beta-1-globulin known as transmanganin. The importance of vitamin B_6 as a participant in enzymatically-catalyzed reactions of tryptophan metabolism is well-recognized. Feeding high levels of magnesium may also affect tryptophan metabolism, possibly through its involvement in vitamin B_6-catalyzed reactions. Two rather dissimilar studies suggest that magnesium and pyridoxine metabolism may be related. Thus, there is evidence that metabolism of tryptophan and vitamin B_6, vitamin B_6 and magnesium, and tryptophan and magnesium may be related. Only one study is reported here which directly links tryptophan, vitamin B_6 and magnesium metabolism, but the probability that such a relationship exists seems likely, particularly from the evidence that metal chelates of pyridoxylidene amino acids may function as intermediates in biological reactions (Christensen, 1957; Christensen and Riggs, 1956; Matsuo, 1957; Davis *et al*. (1961); Pal and Christensen, 1959).

METHODS AND PROCEDURES

General Plan

This investigation was designed to test the effects of diets containing different levels of manganese or magnesium and of magnesium with selected combinations of vitamin B_6 and tryptophan on hepatic nucleic acids, splenic nucleic acids, weight gain, immune response, and femur levels of manganese and magnesium.

The entire study was composed of 6 experiments, 1 through 6. Weanling, male rats of the Wistar strain were used in the study except in experiment 4 which employed 200-gram, male rats, also of the Wistar strain. Rats for experiments 1-5 were obtained from the stock colony of the Iowa State University Food and Nutrition laboratory. Animals for experiment 6 were obtained from the commercial breeder[1] from which the original animals in the colony had been produced.

Diets were fed ad libitum in all experiments, and rats were immunized with sheep red blood cells and bled according to plans designated under the specific experiments. Blood was withdrawn at autopsy, and the serum was separated and frozen for analysis at a later time. Kidneys and testes were excised and weighed; spleens, livers, and femurs were excised, weighed and frozen until analyses were performed.

The overall experimental plan is summarized in Table 1; details of individual experiments are recorded under their respective sections.

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[1]Simonsen Laboratories, White Bear Lake, Minnesota.

Table 1. Summary of experimental plans[a]

Experiment no.	Duration	Group no.	No. rats/ group	Diets fed (No. days diet was fed)	Bleeding days after immunization
	days				
1	71	I	10	Control[b] (28) ⟶ Control[b] (43)	5, 7, 14
		II	10	Control[b] (28) ⟶ 3X Mn[b,c] (43)	
		III	10	Control[b] (28) ⟶ 3X Mn, Mg, Fe, Cu[b,c] (43)	
		IV	10	Mn-free[b] (28) ⟶ Mn-free[b] (43)	
2	120	V	10	Control[d] (120)	5, 7
		VI	10	Mn-free[d] (120)	
3	45	VII	10	Control[e] (45)	5, 9
		VIII	10	Mn-free[e] (45)	
		IX	10	Mg-free[e] (8) ⟶ ½Mg[c,e] (37)	
4	38	X	10	Control[e] (38)	5, 9
		XI	10	Mg-free[e] (38)	
5	55	XII	8	Control[e] (55)	5, 7
		XIII	8	Mn-free[e] (55)	
		XIV	8	5X Mn[c,e] (55)	
		XV	8	5X Mn, Mg, Fe, Cu, Zn[c,e] (55)	

6	46	XVI	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ 5½Mg[c,e] (11)	5, 9
		XVII	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ 5X B_6[e,g] (11)	
		XVIII	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ 5X try[e,h] (11)	
		XIX	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ 5½Mg[c] + 5X B_6[e,g] (11)	
		XX	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ 5X try[h] + 5X B_6[e,g] (11)	
		XXI	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ 5½Mg[c] + 5X try[h] + 5X B_6[e,g] (11)	
		XXII	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ Mn = 5X Mg[e,i] (11)	
		XXIII	10	Control[e,f] ⟶ ½Mg[c,e] (35) ⟶ ½Mg[c,e] (11)	
		XXIV	10	Control[e,f] ⟶ 21.5% Casein (35) ⟶ 21.5% Casein (11)	
		XXV	10	Control[e,f] ⟶ Control[e] (35) ⟶ Control[e] (11)	

[a] All diets fed ad libitum.

[b] Contained 15% protein: 10% from casein + 5% from corn. Cornstarch was the source of CHO.

[c] Numbers refer to multiples or fractions of the amounts of the nutrients recommended for the rat by the National Academy of Sciences-National Research Council (1962).

[d] Contained 15% protein from casein. Dextrose was source of CHO in this and all subsequent expts.

[e] Contained 30% protein from casein.

[f] Control diet fed until each rat reached a body weight of 80 ± 5 grams.

[g] A weight of pyridoxine HCl (General Biochemicals Inc., Chagrin Falls, Ohio) amounting to 5 times the amount recommended for the rat by the National Academy of Sciences-National Research Council (1962) was made up in 20% ethanol and given as a daily supplement in addition to the complete vitamin mix which contained adequate pyridoxine.

[h] A weight of DL-tryptophan (General Biochemicals Inc., Chagrin Falls, Ohio) equivalent to 5 times the amount recommended for the rat by the National Academy of Sciences-National Research Council (1962) was added to the diet.

[i] A weight of manganese sulfate (Mallinckrodt Chemical Works, St. Louis, Missouri) was substituted for 5 times the recommended amount of magnesium (N.A.S.-N.R.C. (1962)) on an equivalent-weight basis.

Care of Animals

Rats in all experiments were housed individually in stainless-steel, wire-mesh cages on racks in a temperature- and humidity-controlled room. All rats received deionized water[1] and their respective diets ad libitum. They were weighed 2 to 3 times a week and weekly food intake records were kept.

Cages and racks were changed weekly. Water bottles were rinsed with deionized water before filling them. Food jars and the paper towels placed on the trays beneath the cages to catch spilled food were changed and weighed 2 to 3 times weekly. Liquid vitamin supplements in quantities of 1 ml were pipetted daily into separate containers to which 2 drops of cod-liver oil and 2 drops of alpha-tocopherol solution (Table 2) were added immediately prior to feeding.

Composition and Preparation of Mineral Mixes and Diets Used

Composition of the mineral mix used for control diets in all experiments (Table 3) was based upon the minerals known to be essential for the rat and in the concentrations recommended by the Committee on Animal Nutrition, National Academy of Sciences-National Research Council (1962). Mineral mixes for the experimental diets were similar except for varying the concentrations of test minerals in individual experiments as indicated. Minerals used were weighed, ground together in a mortar and pestle, and mixed thoroughly by shaking in a glass jar.

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[1]Barnstead mixed-bed cartridge, Barnstead Mfg. Co., Boston, Massachusetts.

Table 2. Composition of the vitamin mixture

Vitamin[a]	Dosage per day	Vitamins per 2,000 doses
	mcg	mg
In 20% Ethanol:		
Thiamine HCl	20.0	40.0
Riboflavin	39.0	78.0
Pyridoxine HCl	20.0	40.0
Folic acid	20.0	40.0
Calcium pantothenate	97.0	194.0
Para-aminobenzoic acid	97.0	194.0
Vitamin B_{12}	0.2	0.4[b]
Biotin	2.0	4.0[c]
Niacin	200.0	400.0
Inositol	2.4 mg	4.8 g
Choline HCl	4.8 mg	9.6 g
In addition:		
dl-α-tocopherol in oil[d,e]	1.0 mg	2.0 g[f]
Cod-liver oil[d]	50.0 mg[g]	

[a]All vitamins were obtained from General Biochemicals, Chagrin Falls, Ohio, with the exception of niacin which was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Cod-liver oil was obtained from Squibb Laboratories, Detroit, Michigan.

[b]400 mg of 0.1% vitamin B_{12} triturated in mannitol.

[c]400 mg of 1% biotin triturated in dextrin.

[d]Medicine droppers were calibrated so that a 2 drop dose equalled 50 mg of solution.

[e]Mazola corn oil, Best Foods, Englewood Cliffs, New Jersey.

[f]In 98 g Mazola corn oil, Best Foods, Englewood Cliffs, New Jersey.

[g]Provides 42.5 and 4.25 USP units of vitamins A and D, respectively, per rat per day.

Table 3. Composition of mineral mix used in control diets[a,b]

Components	Formula	Amount
		g/kg diet
Calcium phosphate (dibasic)[c]	$CaHPO_4$	20.3687
Copper sulfate (anhydrous)[c]	$CuSO_4$	0.0126
Magnesium sulfate (anhydrous)[c]	$MgSO_4$	1.9798
Potassium iodide[d]	KI	0.0003
Potassium phosphate (dibasic)[e]	K_2HPO_4	4.0094
Sodium chloride[c]	NaCl	1.2711
Zinc sulfate[d]	$ZnSO_4 \cdot 7\ H_2O$	0.0528
Iron sulfate[f]	$FeSO_4 \cdot 7\ H_2O$	0.1244
Manganese sulfate[d]	$MnSO_4 \cdot H_2O$	0.1538

[a]Amounts of salts used were based on amounts recommended for the rat by the National Academy of Sciences-National Research Council (1962).

[b]Mineral mixes used in the various experimental diets differed only in the total amount of the element varied as indicated in the specific diet; minerals were added or deleted at the expense of carbohydrate.

[c]J. T. Baker Chemical Company, Phillipsburg, New Jersey.

[d]Mallinckrodt Chemical Works, St. Louis, Missouri.

[e]Matheson, Coleman and Bell, Norwood, Ohio.

[f]Allied Chemical and Dye Corp., New York, New York.

Compositions of the control diets used are given in Table 4a. Experimental diets used in each study varied from the control only in the mineral content. In some cases, the proportion of mineral mix in the diet increased by weight at the expense of the carbohydrate used. Control and diets calculated to be low in either mineral were analyzed for manganese

Table 4a. Compositions of the control diets used

Ingredients	Experiment 1	Experiment 2	Experiments 3-6
		g/100 g diet	
Cornstarch[a]	20.27	--	--
Glucose[b]	--	73.33	57.12
Mineral mix[c]	2.80	2.80	2.80
Non-nutritive fiber[a]	2.00	2.00	2.00
Corn oil[d]	5.00	5.00	5.00
Ground yellow corn[e]	58.62	--	--
Casein[f]	11.11	16.67	32.88
DL-methionine[a]	0.20	0.20	0.20

[a]General Biochemicals Inc., Chagrin Falls, Ohio.

[b]Dextrose, technical monohydrate, General Biochemicals Inc., Chagrin Falls, Ohio.

[c]Composition given in Table 3.

[d]Mazola, Best Foods, Englewood Cliffs, New Jersey.

[e]General Biochemicals Inc., Chagrin Falls, Ohio. Corn analyzed previously by macro-kjeldahl procedure contained 8.53% protein.

[f]General Biochemicals Inc., Chagrin Falls, Ohio. Mfg. analysis reported most lots contained 90% protein.

and magnesium using atomic absorption spectrophotometry; respective values are given in Table 4b. Fat, fiber and protein concentrations were identical for control and test diets within each experiment.

Diets were mixed in quantities of 10 kilograms in the stainless-steel bowl of a Hobart mixer and stored in polyethylene containers at -20°C. until used. Working quantities of 1-2 kilograms were removed from the freezer and stored at 4°C. for convenient daily use.

Table 4b. Manganese and magnesium contents of diets fed during all experiments as analyzed using atomic absorption spectrophotometry

Experiment #	Diet	Mn[a] mg/100g diet	% of NRC requirement provided by diet	Mg[b] mg/100g	% of NRC requirement provided by diet
1	Control	5.0	100		
	Mn-free	0.3	6		
2	Control	5.2	104		
	Mn-free	0.1	2		
3	Control	4.7	94	48.0	120
	Mn-free	0.1	2		
	Mg-free			1.0	2.5
	¼ Mg			14.2	35.5
4	Control			48.0	120
	Mg-free			1.0	2.5
5	Control	4.7	94		
	Mn-free	0.1	2		
6	Control	5.3	106	39.6	99
	¼ Mg			12.0	30

[a] NRC requirement = 5 mg/100g diet.

[b] NRC requirement = 40 mg/100g diet.

Immunization

Rats in all experiments were anesthetized with ether and then immunized with 1 ml of a 2% suspension of sheep red blood cells (SRBC) in 0.85% saline injected through a lateral caudal vein with a 25-gauge sterile needle. Before the suspension for injection was prepared, the cells[1] were washed with 0.85% saline until a clear wash was obtained. Cells were gently mixed with 0.85% saline in a graduated centrifuge tube and centrifuged for 5 minutes at 2,500 rpm. This procedure was repeated until a clear wash was obtained. Following the final wash, the suspension was centrifuged for 8 minutes, and cells were diluted to give the 2% suspension used for immunization.

Autopsy Procedure

Rats were sacrificed using an intraperitoneal injection of 0.5-1.0 ml of sodium pentobarbital solution.[2] A midline incision was made to the diaphragm to expose the abdominal cavity after reflexes were lost. Blood was withdrawn from the abdominal aorta using a sterile 20-gauge needle on a 20-ml syringe. If this procedure failed, the thoracic cavity was opened, and blood was withdrawn from the heart. The plunger of the syringe was removed, and the blood was poured into a 15-ml centrifuge tube and allowed to clot at room temperature (26°C.) for 30 minutes. It was then stored in a refrigerator until the day's autopsies were completed.

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[1] Baltimore Biological Laboratories, Westchester, Pennsylvania.

[2] 50 mg/ml, Abbott Laboratories, North Chicago, Illinois.

At this time, the blood samples were centrifuged for 15 minutes at 2,000 rpm. Sera were collected, transferred to clean centrifuge tubes and re-centrifuged for 15 minutes at 2,000 rpm. Samples were stored at -20°C. in glass vials until analyzed later. Spleens, livers, kidneys, and testes, if taken, were excised, blotted on absorbent paper, weighed, wrapped in aluminum foil, and frozen in liquid nitrogen. Thymuses were removed and treated similarly in experiment 6. Left femurs were removed, cleaned, weighed and wrapped in aluminum foil. All tissues were stored at -20°C. until analysis later.

Experimental Conditions

Experiment 1

The purposes of experiment 1 were to determine the effects of a) concentration of manganese in the diet, and b) ratio of manganese to other key minerals in the diet on the immune response and growth of young, male rats.

Four groups of 10 male, weanling rats (I.S.U. Wistar) were employed. Three groups (I-III) were fed the control diet containing manganese at the levels recommended by the Committee on Animal Nutrition, National Academy of Sciences-National Research Council for 28 days prior to beginning experimental diets. At the end of the 28 day period, animals in Group I remained on the control diet, while animals in Groups II and III were changed to diets containing 3 times the NRC recommended levels of manganese or 3 times the NRC recommended levels of manganese, iron, magnesium, and copper, respectively, for the remainder of the experiment (43 days). Rats in Group IV received a diet with no manganese added for

the entire 71 day period. All diets (Tables 3 and 4a) were fed ad libitum and contained 15% protein (10% from casein; 5% from yellow corn). Cornstarch was the source of carbohydrate, and the rats received daily vitamin supplements (Table 2). On the 57th day (29 days after Groups II and III were put on their experimental diets), all rats were immunized with 2% SRBC. They were bled from a tail nick on days 62 and 64 and sacrificed on day 71. Sera were collected and frozen for analysis later. Livers, spleens, and kidneys were removed, weighed and frozen as described in the autopsy procedure.

Experiment 2

The purposes of experiment 2 were a) to develop a dietary regimen which would produce manganese deficiency in rats and b) to compare the immune responses of manganese-deficient rats with those of control rats.

Two groups of 10 male, weanling rats were fed either the control diet (Group V), containing the level of manganese recommended by the NRC, or a diet with no added manganese (Group VI) for 120 days (Tables 3 and 4a). Both diets contained 15% protein from casein. Dextrose was the source of carbohydrate in this and all subsequent experiments. Each rat received vitamin supplements (Table 2). Rats were immunized on experimental day 113, bled on day 118, and sacrificed on day 120. Sera were collected and frozen for analysis later. Livers, spleens, kidneys and testes were removed, weighed and frozen as described in the autopsy procedure.

Experiment 3

The purposes of experiment 3 were a) to determine whether increasing dietary protein from 15% to 30% would improve growth of rats fed dextrose-base diets and b) to compare the immune responses of rats fed manganese- and magnesium-deficient diets with each other and with those of control rats.

Three groups of 10 male, weanling rats were fed a control diet adequate in manganese and magnesium (Group VII), a diet with no manganese added (Group VIII), or a magnesium-deficient diet (¼ of requirement added) (Group IX). Group IX was fed a "magnesium-free" diet (no added magnesium, Table 4b) for 8 days (experimental days 1-9). On day 9 these rats were changed to the "low-magnesium" diet containing ¼ of the magnesium recommended for rats by the NRC (Table 4b); this diet was fed for the remainder of the experiment (days 9-45). All diets (Tables 3 and 4a) contained 30% protein from casein. Dextrose was the source of carbohydrate. Each rat received daily vitamin supplements (Table 2). Animals were weighed daily for the first 7 days of the experiment and then 2-3 times weekly thereafter. Food intakes were recorded weekly. Animals were immunized on day 36, bled on day 41 and sacrificed on day 45. Sera were collected for analysis later. Livers, spleens, kidneys, testes and left femurs were removed, weighed, and frozen as described in the autopsy procedure.

Experiment 4

The major purpose of experiment 4 was to determine the effects of feeding an almost magnesium-free diet (Table 4b) on the immune response of older animals having greater magnesium reserves than younger animals.

Two groups of 10 male, 200-gram rats were fed a control diet (Group X) or a diet containing no added magnesium ("magnesium-free") (Group XI) for 38 days. Diets (Tables 3 and 4a) contained 30% protein from casein. Dextrose was the carbohydrate source. Rats received daily vitamin supplements (Table 2). On the 29th day, rats were immunized, bled on day 34 and sacrificed on day 38. Sera were collected for analysis later. Livers, spleens, kidneys, testes and left femurs were removed, weighed, and frozen as described in the autopsy procedure.

Experiment 5

The purposes of experiment 5 were a) to determine the effect of level of manganese fed in the diet on the immune response and b) to determine what effect the ratio of manganese to other key minerals in the diet has on the immune response of young male rats.

Four groups of 8 male, weanling rats were fed a control diet adequate in manganese (Group XII), a diet with no added manganese (Group XIII), a diet containing manganese at a level of 5 times the amount recommended for rats by the NRC (Group XIV), or a diet containing manganese, magnesium, iron, copper and zinc at levels of 5 times the amounts recommended for rats by the NRC (Group XV) (Tables 3 and 4a) for 55 days. Diets contained 30% protein from casein. Dextrose was the carbohydrate source. Rats received daily vitamin supplements (Table 2). Rats were

weighed daily for the first 7 days and then 2-3 times weekly thereafter. On the 48th day all rats were immunized, bled on day 53 and sacrificed on day 55. Sera were collected for analysis later. Livers, spleens, kidneys, testes and left femurs were removed, weighed and frozen as described in the autopsy procedure.

Experiment 6

The purposes of experiment 6 were a) to investigate interrelationships in magnesium, manganese, tryptophan and vitamin B_6 metabolism in rats fed low-magnesium diets and the effects these interrelationships have on their immune responses and b) to determine whether the depression in antibody formation in magnesium-deficient rats is due to the deficiency of magnesium or whether it could be caused by a decrease in protein intake resulting from a lower food intake.

Ten groups of 10 male, weanling rats obtained from a commercial breeder were employed in this experiment. Upon arrival all rats were given the control diet (Table 4a) containing 30% protein from casein until they reached a body weight of 80 $\pm$ 5 grams. Individual rats were then assigned to a 30% protein, low-magnesium diet (25% of the amount recommended by the NRC), to a protein-restricted, magnesium-adequate diet (21.5%[1] protein), or to a 30% protein, magnesium-adequate control diet for 35 days. Casein was the source of dietary protein. Thus, during this 35-day period, 80 rats were maintained on the low-magnesium

[1]Amount was set at 21.5% because the magnesium-deficient rats in previous experiments consumed about 72% as much food as controls.

diet, 10 rats on the protein-restricted diet, and 10 rats on the control diet. Rats received daily the standard vitamin supplements (Table 2) in addition to their diets.

On day 35 rats fed control (Group XXV) and protein-restricted (Group XXIV) diets were continued on these regimens for an additional 11 days. The rats maintained on the low-magnesium diet were divided into 8 groups of 10 and fed experimental diets for the 11 additional days as follows:

Group XVI	5¼ times the recommended amount of magnesium
Group XVII	low-magnesium diet + vitamin B_6
Group XVIII	low-magnesium diet + tryptophan
Group XIX	5¼ times the recommended amount of magnesium + vitamin B_6
Group XX	low-magnesium diet + tryptophan and vitamin B_6
Group XXI	5¼ times the recommended amount of magnesium + tryptophan and vitamin B_6
Group XXII	low-magnesium diet + manganese (139.01g Mn $SO_4 \cdot H_2O$) equivalent to 5 times the recommended amount of magnesium
Group XXIII	low-magnesium diet.

All diets contained 30% protein from casein. Tryptophan and vitamin B_6 supplements were at the level of 5 times the amounts recommended for the rat by the NRC. Vitamin B_6 supplement contained 5 times NRC recommendations per milliliter, in 20% ethanol. During the 11 day period, the regular vitamin solution (Table 2) was diluted with an equal volume of either vitamin B_6 solution or of 20% ethanol, and 2 ml was given daily. This equalized the ethanol intakes and eliminated the necessity of making 2 vitamin mixtures. Rats were immunized on day 37 (2 days after

beginning the experimental diets). They were bled on day 42, and sacrificed on day 46. Sera were collected for analysis later. Livers, spleens, kidneys, testes, thymuses and left femurs were removed, weighed and frozen as described in the autopsy procedure.

Immunochemical Analyses

Agglutinin and hemolysin activities of the immune sera were determined using a semi-micro titration method described by Patten (1970). Special microtiter equipment[1] consisting of transparent plates with 8 rows of 12 depressions for making dilutions, metal loops designed to contain 0.025 ml of serum, and metal-tipped plastic pipettes calibrated to deliver 0.025 ml-drops of the reagents were used. Samples were analyzed in duplicate on the same day and were re-analyzed whenever titers of the duplicates did not agree within one dilution.

Agglutinin

Using the dropping pipette calibrated to deliver 0.025 ml, 2 drops of buffer solution of pH 7.4 (Pillemer et al., 1956) were added to the first depression of each row of the transparent plate. One loop of serum was mixed with the buffer in each of the depressions in the first row. This row of diluted serum was covered with a strip of plastic tape to prevent evaporation prior to incubation of the plate for 20 minutes at 56°C. to inactivate the complement. After incubation, 1 drop of buffer was added to each of the remaining depressions on the plate; two-fold

[1]Cooke Engineering Company, Alexandria, Virginia.

serial dilutions were made across the plate from the original 1:3 dilution using the special holder equipped with the short-stemmed loops for transfer and mixing. One drop of 1.5% SRBC (washed as described in the immunization procedure but diluted to 1.5% with buffer) was added to each depression, and the plate was tapped gently to mix the contents of the depressions. This suspension was then allowed to sit for 1½ hours at room temperature. The end-point was recorded as the highest dilution of serum showing positive agglutination. Results were expressed as the logarithms of the reciprocals of these dilutions.

Hemolysin

Before determining serum hemolysin activity, the strength of guinea pig complement[1] needed for the titration procedure was determined. Reconstituting fluid was used to rehydrate the lyophylized guinea pig serum and a 1:10 dilution was made with $MgCl_2$-saline solution. One or 2 drops of Pillemer's buffer were added to the first depressions of rows 1 and 2 and rows 3 and 4 respectively of the microtiter plate. One drop of buffer was added to each of the remaining depressions in the 4 rows. One loop of the 1:10 diluted complement was added to the first depression of these 4 rows, effecting a 1:20 initial dilution of complement in rows 1 and 2 and a 1:30 dilution in rows 3 and 4. Serial dilutions were made across the 4 rows. One drop of 2% SRBC in Pillemer's buffer was added to each depression, followed by 1 drop of 0.2% rabbit antiserum[2] in Pillemer's

[1]Bacto-complement, Difco Laboratories, Detroit, Michigan.

[2]Bacto-anti-sheep hemolysin, Difco Laboratories, Detroit, Michigan.

buffer. The solutions were mixed by tapping the plate lightly. It was then covered with aluminum foil and incubated for 40 minutes in a 37°C. water bath. The end-point was taken as the dilution at which half of the SRBC were lysed as judged visually. Four times the concentration of complement that produced the end-point was used for the hemolysin titrations of the rat sera.

Sera were usually initially diluted 1:4 on the plate before making serial dilutions in drops of buffer across the plate. One drop of 2% SRBC (previously washed as described in the immunization procedure) in Pillemer's buffer and a drop of appropriately diluted complement were added to each depression. The suspension in the depressions was mixed by tapping the plate lightly. The plate was then covered with aluminum foil and incubated for 40 minutes in a 37°C. water bath. The end-point was recorded as the dilution at which half of the SRBC were lysed. Results were expressed as the logarithms of the reciprocals of these dilutions.

Chemical Analyses

RNA in livers and spleens

On the evening preceding the day RNA analyses were to be performed, livers or spleens were homogenized and filtered. Livers and spleens were removed from the freezer, minced with scissors while partially frozen, homogenized with cold, deionized water and made to volumes of 100 ml and 50 ml, respectively, with cold, deionized water. Volumetric flasks of diluted homogenates were then packed in ice. The homogenates were filtered through 2 layers of cheese cloth. Aliquots of the filtrates,

containing 0.10-0.14 g liver or 0.04-0.06 g spleen, were pipetted in triplicate into 15-ml centrifuge tubes suspended in a 0°C. constant-temperature, water-glycerol bath, diluted to 5 ml with cold, deionized water, and covered until analysis using a modification of the procedure described by Munro and Fleck (1966).

The following morning, 2.5 ml of cold 0.6 N perchloric acid (PCA) was added to each tube to precipitate the nucleic acids and proteins; mixtures were allowed to stand for 10 minutes at 0°C. before they were centrifuged for 10 minutes at approximately 2,300 rpm in a cold room. The resulting supernatant fractions were discarded and the tubes drained briefly onto absorbent paper. The precipitates were then washed twice with cold 0.2 N PCA, allowing a 10 minute waiting period in the 0°C. bath before each centrifugation. Afterward the supernatants were discarded and the tubes drained onto absorbent paper as before.

The precipitates were then mixed with 4 ml of 0.3 N KOH; this mixture was incubated in a 37°C. water bath for 1 hour to hydrolyze the RNA. After the incubation period the samples were cooled in the 0°C. bath for 15 minutes, or until their temperatures reached 0°C. The protein and DNA were precipitated by adding 2.5 ml of cold 1.2 N PCA and allowing the mixture to stand for 10 minutes in the cold bath. These mixtures were centrifuged in the cold, and the supernatants containing the hydrolyzed RNA were decanted into 50-ml or 25-ml volumetric flasks for the liver and spleen analyses, respectively. The precipitates were washed twice with 5 ml of 0.2 N PCA, allowing a 10-minute waiting period before each centrifugation, and the washings were

added to the flasks. Enough 0.6 N PCA (5 ml and 2.5 ml for livers and spleens, respectively) was added to each flask to produce a final solution of ribonucleotides in 0.1 N PCA after the contents of the flasks were brought to volume with deionized water.

RNA content of these solutions was measured by UV absorption at 260 mμ. Munro and Fleck (1966) found that an extinction of 1.000 at 260 mμ is equivalent to 32μg RNA/ml for rat liver. This value was used for calculating spleen RNA content also. Readings were also taken at 280 and 310 mμ to check for possible protein contamination in all samples by calculating the (260 - 310)/(280 - 310) ratios. Any sample with a ratio below 1.34 was not included in calculating the RNA content of the particular tissue (Munro and Fleck, 1966).

DNA in livers and spleens

DNA contents of livers and spleens were determined on the precipitates which were left after the supernatants containing RNA were transferred to volumetric flasks. These precipitates were mixed with 0.3 N KOH (5 ml or 7 ml for liver or spleen analyses, respectively) and allowed to stand overnight in a refrigerator to obtain the DNA in a soluble form for analysis. After these alkaline mixtures were centrifuged the next day, the supernatants were transferred to 25 ml or 50 ml volumetric flasks for the liver and spleen analyses, respectively. The precipitates were washed with 0.3 N KOH (3.5 ml and 5 ml for liver and spleen, respectively) and centrifuged for 5 minutes, and the supernatants transferred to the volumetric flasks in use. An additional 5 ml of 0.3 N KOH was added to each volumetric flask used in the spleen

analyses. The DNA solutions in the flasks were brought to volume with deionized water to give final solutions of DNA in 0.1 N KOH.

Two-milliliter aliquots of the DNA solutions from the flasks were pipetted into clean, screw-capped culture tubes; 1 ml of 0.04% indole and 1 ml of 2.5 N HCl were added to all tubes, and each was mixed with an electric test-tube mixer. The tubes were capped loosely, placed in a boiling water bath for 20 minutes, removed and cooled immediately in cold, running water for about 10 minutes. Solutions were then extracted 3 times with 4 ml chloroform, centrifuging for 5 minutes to give a clear water-phase after each extraction. The chloroform layer was removed each time using a long, disposable pipette attached to a vacuum flask connected to the water supply. A series of standard solutions (usually 10, 20, 30, 40 μg/2 ml and 60, 80, 100 μg/2 ml if needed) were prepared from salmon sperm DNA[1] and treated in the same way as the samples. Absorbancies of the aqueous phases containing the DNA were read at 490 mμ. Readings were corrected by subtracting the readings of blanks which had been similarly handled. DNA values of the samples were calculated from a standard curve prepared from the absorbancy values obtained with the known solutions.

Separation of antibody by column chromatography

Gamma globulin extraction Gamma globulin was extracted and antibodies were separated using rat sera from experiments 3 and 4 and selected groups from experiment 6. A modification of the method

[1]Calbiochem, Los Angeles, California.

described by Friedman (1958) was used for extraction of gamma globulin. Serum gamma globulin was precipitated using a solution 1.4 M (33% saturated) in ammonium sulfate and 0.5 M in sodium chloride with a pH of 6.4. The protein was precipitated in the cold, and the supernatant was removed after centrifugation.

Three-tenths milliliter of serum was carefully overlaid on 5.7 ml of the ammonium sulfate reagent in a 14-ml polypropylene centrifuge tube. The tubes were capped, inverted gently about 6 times to mix, and placed in a container of ice for 15 minutes. At the end of this period they were centrifuged[1] at -4°C. for 30 minutes at 15,000 rpm. The supernatants were discarded, and the precipitates were dissolved in a small amount of phosphate buffer,[2] pH 7.8. These solutions were transferred to 1-ml volumetric flasks using disposable pipettes. The centrifuge tubes were washed with small quantities of the phosphate buffer, and the washings were transferred to the volumetric flasks. After making the solutions to volume with buffer, they were transferred to ½-dram vials, covered with plastic film, capped and stored at -20°C. until used.

Column-packing procedure and antibody measurement Column chromatography carried out at room temperature was used to separate antibody from other gamma globulin in the solution prepared as described in the preceding section. A column,[3] 30 cm in length and 1.5 cm in

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[1]Sorvall superspeed automatic refrigerated centrifuge, model RC2-B, rotor type SE-12, Sorvall, Newtown, Connecticut.

[2]Sorensen's buffer, Frankel and Reitman (1963), pp. 205-206.

[3]Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

diameter, was used with the corresponding eluant reservoir.[1] Parts of the column were assembled and "O" rings were greased[2] to prevent leakage at connections. The assembled column was clamped to the framework of an automatic fraction collector, and the delivery tube was connected to a device which counted drops delivered from the column into the tubes below. Initially, the delivery tube was clamped to stop the flow, and the column was filled to about 1/3 of its capacity with deionized water. A gel slurry was prepared using approximately 50 ml deionized water and 50-60 ml Sepharose 6B[3] and then deaerated under a vacuum of about 25 psi. The eluant reservoir was fitted to the top of the column as an extension tube using the conical adapter. The gel slurry was poured into the column with one addition, the delivery tube was unclamped, and the gel was allowed to settle into an evenly-distributed bed with a clear space of about 2 cm at the top of the column which was filled with Sorensen's buffer as soon as the deionized water level reached the gel surface. The eluant reservoir (Mariotte flask) was assembled and connected to the column by capillary tubing. Eluant flow-rate was controlled by pressure changes caused by raising and lowering the Mariotte flask. The column was equilibrated by allowing it to run overnight at a flow-rate of 3 drops per minute.

The same gel was used for experiments 3 and 4; a new bed was prepared for experiment 6. The volume outside the gel matrix, referred

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[1]Eluant reservoir R-15 (410 ml), Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

[2]High-vacuum grease, silicone lubricant, Dow-Corning, Midland, Michigan.

[3]Sephadex with 6% agarose added, Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

to as the void volume, was approximately 15 ml for the first bed and was determined by eluting 0.5 ml of a 0.3% blue dextran[1] solution diluted with 0.02% sodium chloride; fractions were read at 280 mμ. The first fraction giving a significant reading was not included in the void volume. The void volume for the second gel was determined in the same way and amounted to about 12.1 ml. The total bed volume, that occupied by the gel beads plus the void volume, was determined for both gels by eluting 0.5 ml of a 4% formaldehyde solution from the column. Fractions were tested with Schiff reagent for aldehydes,[2] and the tube giving the most intense pink color as judged visually was designated the end of the bed volume. Bed volumes were about 54.5 ml and 42.4 ml for the first and second gels, respectively.

For antibody separation, 0.5 ml of the gamma globulin solution, prepared as described previously, was added to the top of the column. The reservoir of phosphate buffer was connected, and the sample was eluted at a flow rate of 3 drops per minute. Usually, 38 fractions of 1.5 ml (50 drops) were collected for each sample. The protein content of each fraction was estimated by recording the UV absorptions at 280 mμ and comparing them with a standard curve prepared from readings taken using standard gamma globulin[3] solutions. Protein concentration in the gamma globulin solutions which were applied to the column were determined in the same way.

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[1]Molecular weight 2 x 10^6, Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

[2]Frankel and Reitman (1963), p. 347.

[3]Rat gamma globulin, Fraction II, Pentex, Inc., Kankakee, Illinois.

Mineral analyses

Diets and femurs were analyzed for manganese and magnesium by atomic absorption spectrophotometry.[1] Aqueous standards of both metals were made. Maximum absorbancy readings occurred at 278.0 mμ for manganese and 283.9 - 284.5 mμ for magnesium when these standards were tested.

Diets Samples of the diets, taken at the time they were prepared, were stored in polyethylene containers at -20°C. until they were analyzed. At the time of analysis, diets were brought to room temperature and 2-7 gram, triplicate portions of each were weighed into porcelain crucibles which had been brought to constant weight previously. Crucibles containing the diets were placed in a muffle furnace where samples were dried for 2 hours at 100°C. After the drying period, the temperature of the furnace was raised by 50°C. increments each half-hour until a final temperature of 540°C. was reached. Samples were ashed at 540°C. for 8-10 hours. Klinking occurred in the first ashing, so a second heating was necessary. The cooled ash was dissolved in 7 drops of 70% HNO_3 and dried for 2 hours at 100°C. The temperature of the furnace was raised immediately to 540°C. and samples were re-ashed for 8-10 hours. Empty crucibles were carried through all procedures for preparation of blanks.

The ash was cooled, weighed, and then dissolved in 10 ml of 0.1 N HCl and made to a final volume of 25 ml with deionized water. Ash solutions were stored in small, polyethylene bottles until they were

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[1]Unicam Model Sp. 90 Atomic Absorption Spectrophotometer. Philips Electronic Instruments, Mount Vernon, New York.

analyzed. Manganese determinations were made on these solutions. For those samples on which magnesium determinations were made, 1-ml aliquots of the ash solutions were diluted to 50 ml with 1% lanthanum acetate[1] prior to reading. Ash solutions from the "magnesium-free" diets were not diluted. Samples were read alternately with standard solutions using an element-specific lamp. Calculations of manganese and magnesium contents were made from standard curves prepared from absorbancy readings of known concentrations of the 2 minerals.

Femurs Femurs were removed from the freezer, and measurements of the lengths and of the diameters in several locations were made prior to ashing. The locations on the femurs where measurements were taken are indicated in Figure 1. D_1 was at the widest point, and D_2 was measured at the base of the third trochanter. D_3 was the thickness at D_2. D_4 was the distance across the condyle region. After measurements were taken and recorded, the femurs were placed in porcelain crucibles which had been brought to constant weight previously. These were placed in a muffle furnace where the femurs were dried for 1 hour at 100°C. After the drying period, the temperature of the furnace was raised by 50°C. increments each half-hour until a final temperature of 600°C. was reached. Samples were ashed at 600°C. for 8-10 hours.

The ash was cooled, weighed and dissolved in either 5 or 10 ml of 0.1 N HCl and made to a final volume of 25 ml with deionized water. Ash solutions were stored in small, polyethylene bottles until they were analyzed. Manganese determinations were made on these solutions. Prior to magnesium determination, 1-ml aliquots of these ash solutions were

[1] K & K Laboratories, Inc., Plainview, New York.

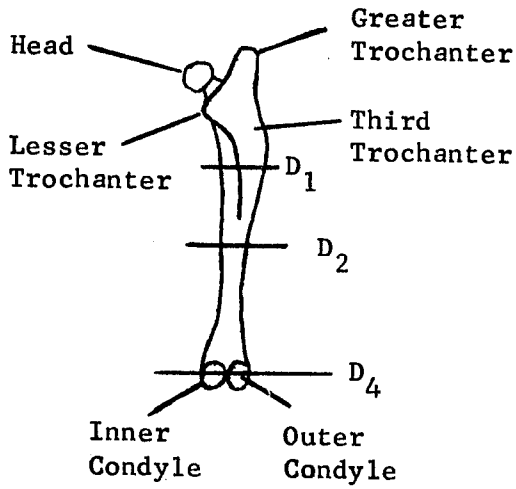

Figure 1. Posterior view of femur showing locations where diameters were measured.

diluted to 50 ml with 1% lanthanum acetate prior to reading. Samples were read alternatively with standard solutions. Calculations of mineral contents were made as described in the preceding section on diets.

Statistical Analysis

The data are presented as means with their standard errors. Analysis of variance and F-tests were used to evaluate differences among groups. The means for some of the groups in experiment 6 were examined using a factorial set of comparisons. Tests of least significant difference were used to identify specific differences among groups within an experiment.

Correlation coefficients between some parameters were also calculated. Differences were considered significant when P was 0.05 or less and highly significant when P was 0.01 or less. Some findings with P between 0.05 and 0.1 were also discussed to evaluate consistency of findings and to identify and describe trends which tended to support or contradict results from other experiments.

RESULTS

This study consisted of 6 discrete experiments. Although some of them were related, the experimental conditions varied enough that it seems expedient to report the results of each experiment individually. Any data which confirm or contradict trends observed across experiments will be considered in the general discussion.

Experiment 1

The purposes of experiment 1 were to determine the effects of a) concentration of manganese in the diet and b) ratio of manganese to other key minerals in the diet on the immune response and growth of young, male rats.

Body weight change, food intake, and food-efficiency

All rats used in this experiment were weaned at approximately 50 g body weight and fed diets as indicated in Table 1. Manganese contents of the control and "manganese-free" diets were 5.0 and 0.3 mg per 100 g diet, respectively, by analysis and provided 100% and 6% of the NRC requirement (Table 4b). Diets for Groups II and III were assumed to contain 3 times the concentration of manganese in the control diet.

Mean body-weight changes, food intakes and food efficiencies for all groups are given in Table 5. Analysis of variance revealed no differences among group means for any parameter during either of the 2 sub-periods (Table 1) or for the overall period. However, when least-significant differences were used for comparing groups, body weight changes and food intakes of Groups II and IV, extremes in manganese

Table 5. Mean body weight changes, food intakes, and food efficiencies in experiment 1

Group	Diet	BWCh[a] Weeks 1-4 g	FI[b] Weeks 1-4 g	FER[c] Weeks 1-4	BWCh Weeks 5-10 g	FI Weeks 5-10 g	FER Weeks 5-10	BWCh Weeks 1-10 g	FI Weeks 1-10 g	FER Weeks 1-10
I	Control	175±4[d]	378±11	0.46±0.01	194±11	817±23	0.24±0.01	368±12	1195±30	0.31±0.01
II	3 x Mn	179±7	387±14	0.46±0.01	210±10[e]	862±30[e]	0.24±0.01	389±14[e]	1247±40[e]	0.31±0.01
III	3 x Complex[f]	180±5	394±7	0.46±0.01	196±11	837±26	0.23±0.01	376±14	1232±31	0.30±0.01
IV	Mn-free	169±7	376±18	0.45±0.01	179±8[e]	769±23[e]	0.23±0.01	348±11[e]	1145±38[e]	0.31±0.01

[a] BWCh = Body weight change.

[b] FI = Food intake.

[c] FER = Food efficiency ratio (wt. change (g)/food intake (g)).

[d] Mean ± S.E.

[e] Different ($P < 0.05$) from each other using test of least significant difference (L.S.D.) F-test showed no differences among groups.

[f] 3 x Mn, Mg, Fe, Cu.

intakes, were significantly different ($P < 0.05$) during the sub-period designated "experimental" as well as for the overall experimental period. Food efficiency ratios for the 2 groups did not differ. The greater weight gain for Group II was a result of an increased food intake during the experimental period and not a result of more efficient food utilization.

Autopsy and organ weights

A pattern similar to that for weight gain was observed for body and organ weights at autopsy (Table 6). Analysis of variance revealed no differences in group means, but the test for least-significant difference showed that autopsy and liver weights were greater ($P < 0.05$) for Group II than for Group IV. The difference in liver weights was not apparent when they were expressed as percentages of body weights. Again, the weight difference was probably a result of the dissimilar food intakes between the groups. Kidney and spleen weights were not different when expressed as absolute values or as percentages of the autopsy weights, although both organs tended to be larger in the heavier animals within each group. Spleen weight was positively correlated ($P < 0.05$) with autopsy weight showing that spleen weight increased or decreased with respective increases or decreases in body weight at autopsy.

Hepatic nucleic acids

Hepatic DNA and RNA were determined for all groups (Table 7). Although the differences were not significant statistically, values for total RNA and DNA in the liver tended to be least for manganese-deficient

Table 6. Mean body and organ weights at autopsy in experiment 1

Group	Diet	Autopsy wt. g	Spleen wt. g	Spleen wt. % AWT[a]	Kidney wt. g	Kidney wt. % AWT	Liver wt. g	Liver wt. % AWT
I	Control	420±12[b]	0.84±0.04	0.20±0.01	2.43±0.10	0.58±0.04	13.89±0.58	3.30±0.06
II	3 x Mn	441±15[c]	0.84±0.04	0.19±0.01	2.63±0.13	0.60±0.02	14.98±1.10[c]	3.37±0.18
III	3 x Complex[d]	428±14	0.82±0.05	0.19±0.01	2.51±0.09	0.59±0.02	14.55±0.56	3.40±0.09
IV	Mn-free	400±12[c]	0.80±0.03	0.20±0.01	2.40±0.10	0.60±0.02	12.63±0.60[c]	3.16±0.14

[a] AWT = Autopsy weight.

[b] Mean ± S.E.

[c] Different ($P < 0.05$) from each other using test of L.S.D. F-test showed no differences among groups.

[d] 3 x Mn, Mg, Fe, Cu.

Table 7. Mean nucleic acid content of livers in experiment 1

Group	Diet	Total RNA mg	mg RNA/ g liver	Total DNA mg	mg DNA/ g liver	RNA/ DNA
I	Control	69.25 ± 3.93[a]	4.97 ± 0.16	30.81 ± 1.32	2.25 ± 0.11	2.26 ± 0.12
II	3 x Mn	69.45 ± 3.53	4.96 ± 0.20	33.41 ± 1.79	2.30 ± 0.14	2.08 ± 0.11
III	3 x Complex[b]	71.14 ± 3.98	4.89 ± 0.18	33.72 ± 1.82	2.33 ± 0.12	2.15 ± 0.15
IV	Mn-free	61.43 ± 2.89	4.95 ± 0.27	30.34 ± 1.38	2.43 ± 0.11	2.05 ± 0.11

[a]Mean ± S.E.

[b]3 x Mn, Mg, Fe, Cu.

animals in Group IV. Livers for this group were also small and, when nucleic acid levels were expressed per gram liver tissue, the trend disappeared. There were no differences in RNA-DNA ratios among groups.

Serum antibody titers

Mean serum antibody titers were reported as the logarithm of serum agglutinin and hemolysin activities (Table 8) as defined in the immunochemical procedure. Serum agglutinin activities tended to be lower on all days for Groups II and IV, which consumed the highest and lowest levels of manganese, but only on day 14 was a significant difference noted. Group II had a significantly lower agglutinin titer than Groups I and III on day 14; Group IV did not differ statistically from the other 3 groups.

Serum hemolysin activity appeared to be relatively unaffected by the conditions of this experiment on days 5 and 7. Group II (high manganese) had significantly ($P < 0.05$) lower hemolysin activity on day 14 than Group IV (low manganese), when tests of least-significant difference were employed.

Experiment 2

The "manganese-free" diet fed in experiment 1 contained by analysis 0.3 mg manganese per 100 g diet, equivalent to 6% of the NRC recommendation for rats (Table 4b). Since this diet had failed to produce clear manganese deficiency symptoms, the purposes of experiment 2 were a) to develop a dietary regimen which would more readily produce manganese deficiency and b) to compare the immune responses of manganese-

Table 8. Mean serum antibody titers 5, 7, and 14 days following immunization in experiment 1

Group	Diet	Agglutinin			Hemolysin		
		Day 5	Day 7	Day 14	Day 5	Day 7	Day 14
		log titer			log titer		
I	Control	1.91 ± 0.20[1,b*]	1.99 ± 0.12[b]	1.63 ± 0.11[c]	2.95 ± 0.29[b]	2.36 ± 0.10[b]	1.62 ± 0.10[c,b]
II	3 x Mn	1.58 ± 0.25[b]	1.79 ± 0.09[b]	1.19 ± 0.12[b]	2.75 ± 0.36[b]	2.37 ± 0.17[b]	1.33 ± 0.13[b]
III	3 x Complex	2.00 ± 0.21[b]	2.02 ± 0.11[b]	1.63 ± 0.12[c]	3.09 ± 0.32[b]	2.39 ± 0.11[b]	1.56 ± 0.08[c,b]
IV	Mn-free	1.84 ± 0.19[b]	1.76 ± 0.12[b]	1.48 ± 0.11[b,c]	3.19 ± 0.26[b]	2.29 ± 0.12[b]	1.71 ± 0.12[c]

[1]Mean ± S.E.

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

deficient rats with those of control rats. The diets were the same as 2 of those in experiment 1, except that dextrose was substituted for cornstarch, and all of the protein was derived from casein.

Body weight change, food intake, and food-efficiency

Weanling, male rats from the Iowa State stock colony were fed a control or "manganese-free" dextrose-base diet (Tables 4a, 4b) for 17 weeks. For comparative purposes, the data for body weight change, food intake and food efficiency were divided into time periods corresponding to the conditioning and experimental sub-periods of experiment 1 (Table 9). There were no differences in any of these parameters between the 2 groups in experiment 2 during any given sub-period or for the overall experimental period. The control diet provided by analysis 52 times as much manganese as the "manganese-free" diet. The control and "manganese-free" diets provided 104% and 2%, respectively, of the NRC recommended value for manganese (Table 4b). In spite of the dissimilar dietary manganese concentrations, the 2 groups grew similarly, had almost equal food intakes and, consequently, the same efficiencies of food utilization. When compared with the animals in experiment 1 over the same time intervals, it is evident that these animals grew much more slowly and utilized their food less efficiently. In fact, these animals took 17 weeks to reach the body weight that animals in experiment 1 reached in 10 weeks.

Table 9. Mean body weight changes, food intakes, and food efficiencies in experiment 2

Group	Diet	BWCh[a] Weeks 1-4 g	FI[b] Weeks 1-4 g	FER[c] Weeks 1-4	BWCh Weeks 5-10 g	FI Weeks 5-10 g	FER Weeks 5-10	BWCh Weeks 11-17 g	FI Weeks 11-17 g	FER Weeks 11-17	BWCh Weeks 1-17 g	FI Weeks 1-17 g	FER Weeks 1-17
V	Control	133 ± 4[d]	364 ± 8	0.37 ± 0.01	155 ± 6	768 ± 31	0.20 ± 0.01	103 ± 7	906 ± 19	0.11 ± 0.01	391 ± 10	2037 ± 40	0.19 ± 0.00
VI	Mn-free	134 ± 5	358 ± 15	0.38 ± 0.01	160 ± 4	775 ± 23	0.21 ± 0.00	93 ± 5	890 ± 17	0.10 ± 0.00	387 ± 9	2023 ± 45	0.19 ± 0.00

[a] BWCh = Body weight change.

[b] FI = Food intake.

[c] FER = Food efficiency ratio (wt. change (g)/food intake (g)).

[d] Mean ± S.E.

Autopsy and organ weights

Mean body and organ weights at autopsy for groups in experiment 2, given in Table 10, were not different between the 2 groups. Of the organs weighed, spleen weight of individual rats was positively correlated ($P < 0.01$) with autopsy weight.

Hepatic nucleic acids

Hepatic RNA and DNA were determined for both groups (Table 11). Groups could not be distinguished on the basis of total RNA or DNA per liver or of nucleic acid per gram of liver tissue. The RNA-DNA ratios were also similar. Thus RNA concentrations per cell were alike for the 2 groups.

Serum antibody titers

Agglutinin and hemolysin log titers are reported in Table 12. Both groups had comparable levels of each antibody 5 and 7 days following immunization. Spleen weights were positively correlated with hemolysin values on day 7 ($P < 0.01$). Agglutinin values on day 5 were positively correlated with hemolysin titers on days 5 and 7 ($P < 0.01$); hemolysin values on days 5 and 7 were positively correlated ($P < 0.01$).

Experiment 3

The diets fed in experiment 2 had failed to produce differences between the groups fed control and "manganese-free" diets (Table 4b) and, in addition, had resulted in slow growth for both groups. It was postulated that 15% protein had been inadequate to support maximum growth rate on dextrose-base diets, and that the animals' reserves were sufficient

Table 10. Mean body and organ weights at autopsy in experiment 2

Group	Diet	Autopsy wt. g	Spleen wt. g	Spleen wt. % AWT[a]	Kidney wt. g	Kidney wt. % AWT	Liver wt. g	Liver wt. % AWT	Testes wt. g	Testes wt. % AWT
V	Control	444 ± 10[b]	0.72 ± 0.03	0.16 ± 0.01	2.67 ± 0.10	0.54 ± 0.06	12.88 ± 0.58	2.89 ± 0.08	3.74 ± 0.19	0.84 ± 0.04
VI	Mn-free	440 ± 9	0.71 ± 0.02	0.16 ± 0.00	2.82 ± 0.09	0.64 ± 0.02	12.28 ± 0.55	2.79 ± 0.10	3.99 ± 0.12	0.91 ± 0.03

[a]AWT = Autopsy weight.

[b]Mean ± S.E.

Table 11. Mean nucleic acid content of livers in experiment 2

Group	Diet	Total RNA mg	mg RNA/ g liver	Total DNA mg	mg DNA/ g liver	RNA/ DNA
V	Control	60.21 ± 1.89[a]	4.72 ± 0.13	24.69 ± 1.81	1.91 ± 0.10	2.54 ± 0.17
VI	Mn-free	61.17 ± 2.57	5.00 ± 0.14	24.86 ± 1.61	2.02 ± 0.11	2.55 ± 0.18

[a]Mean ± S.E.

Table 12. Mean serum antibody titers 5 and 7 days following immunization in experiment 2

Group	Diet	Agglutinin		Hemolysin	
		Day 5	Day 7	Day 5	Day 7
		log titer		log titer	
V	Control	1.45 ± 0.22[a]	1.54 ± 0.18	2.25 ± 0.22	2.57 ± 0.21
VI	Mn-free	1.43 ± 0.21	1.72 ± 0.20	1.98 ± 0.25	2.32 ± 0.21

[a]Mean ± S.E.

to meet physiological needs for manganese when growth was retarded. Consequently, the level of protein was doubled in the third experiment in an attempt to increase growth rate and thereby the speed of depletion of the manganese reserves. It seemed desirable to examine also the consequences of magnesium deficiency on the immune response.

Body weight change, food intake, and food-efficiency

Rats originally fed the "magnesium-free" diet were changed to the "¼-magnesium" diet after 8 days because they were in poor physical condition; on a practical basis, rats were too small to obtain adequate blood samples for antibody titration and separation. Mean body weight change, food intake and food efficiency data are summarized in Table 13. There were differences ($P < 0.01$) in total body weight changes among all groups. Controls gained more weight during the experiment than animals fed the "manganese-free" diet (Group VIII) even though total food intakes of the 2 groups were the same. Although no other signs of manganese deficiency were obvious at the termination of the experiment, it seems likely that the lesser weight gain for Group VIII was a result of insufficient dietary manganese. The "manganese-free" diet provided 2% of the NRC recommendation for manganese, while the control diet provided 94% (Table 4b). Controls also gained more weight per gram of food consumed than Group VIII ($P < 0.01$) during the total experimental period. The differences between Groups VII and VIII were greater over most of the period. Reduced food efficiency may indicate that less manganese was available for growth from reserves as the experiment progressed.

Table 13. Mean body weight changes, food intakes, and food efficiencies in experiment 3

Group	Diet	$BWCh_T$ g [1, 2]	FI_T g [3]	FER_T [4]	FER_1	FER_2	FER_3	FER_4	FER_5	FER_6
VII	Control	199 ± 5 [5,f*]	506 ± 12 [f]	0.39 ± 0.01 [f]	0.38 ± 0.04 [f]	0.51 ± 0.03 [f,g]	0.54 ± 0.03 [f]	0.38 ± 0.04 [f]	0.27 ± 0.05 [f]	0.36 ± 0.03 [f]
VIII	Mn-free	170 ± 5 [g]	508 ± 13 [f]	0.34 ± 0.01 [g]	0.39 ± 0.03 [f]	0.45 ± 0.02 [f]	0.46 ± 0.02 [g]	0.28 ± 0.02 [g]	0.28 ± 0.03 [f]	0.24 ± 0.02 [g]
IX	Mg-deficient	119 ± 11 [h]	346 ± 23 [g]	0.34 ± 0.01 [g]	0.30 ± 0.02 [g]	0.52 ± 0.03 [g]	0.46 ± 0.02 [g]	0.37 ± 0.03 [f]	0.31 ± 0.04 [f]	0.23 ± 0.04 [g]

[1] BWCh = Body weight change.

[2] T = Total.

[3] FI = Food intake.

[4] FER = Food efficiency ratio (wt. change g/food intake (g)); numbered subscripts refer to respective weekly periods.

[5] Mean ± S.E.

* Statistical analysis: means with the same superscripts in a column are not different ($P > 0.05$).

The classic signs of magnesium deficiency (including hyperemia, growth retardation, hair-loss, skin lesions, edema of paws, brown-pigmented mucus in eyes, nose and mouth, and extreme nervousness with convulsions in some animals) were readily apparent in all animals of Group IX by the termination of the experiment. They gained less weight ($P < 0.01$) than either of the other 2 groups. Food intake was also lower ($P < 0.01$) for this group. The lesser food intake accounted for only part of the difference in weight gain between Group IX and the controls, because the efficiency of food utilization was also significantly less ($P < 0.01$). FER was low when the "magnesium-free" diet was fed during the first 8 days and it increased and then gradually declined with the "¼-magnesium" diet fed during the following 37 days. These diets (Table 1) contained 2.5% and 35.5%, respectively, of the NRC recommendation for magnesium, while the control diet provided 120% (Table 4b).

Autopsy and organ weights

Autopsy weights for the 3 groups (Table 14) were affected by the diets in the same way as total weight change, which was reported in the previous section.

Spleens from control animals (Group VII) were heavier ($P < 0.05$) than spleens from manganese-deficient rats in Group VIII, but the difference was removed when weights were expressed on a body-weight basis. By contrast, total weights of spleens from magnesium-deficient (Group IX) and control animals were similar, but the 2 groups were different ($P < 0.01$) when spleen weights were compared on a body-weight basis. Gross splenomegaly in magnesium-deficient animals was easily

Table 14. Mean body and organ weights at autopsy in experiment 3

Group	Diet	Autopsy wt. g	Spleen wt. g	Spleen wt. % AWT[1]	Kidney wt. g	Kidney wt. % AWT	Liver wt. g	Liver wt. % AWT	Testes wt. g	Testes wt. % AWT
VII	Control	247 ± 5[2,c*]	0.61 ± 0.03[c]	0.25 ± 0.01[c]	2.17 ± 0.05[c]	0.88 ± 0.02[c]	9.96 ± 0.29[c]	4.04 ± 0.10[c]	3.26 ± 0.08[c]	1.33 ± 0.05[c]
VIII	Mn-free	217 ± 6[d]	0.50 ± 0.03[d]	0.23 ± 0.01[c]	2.06 ± 0.11[c]	0.95 ± 0.04[c]	9.01 ± 0.51[c]	4.13 ± 0.16[c]	3.26 ± 0.11[c]	1.50 ± 0.04[d]
IX	Mg-deficient	169 ± 11[e]	0.66 ± 0.06[c]	0.39 ± 0.03[d]	2.80 ± 0.13[d]	1.70 ± 0.13[d]	6.77 ± 0.46[d]	4.00 ± 0.11[c]	3.14 ± 0.13[c]	1.88 ± 0.08[e]

[1]AWT = Autopsy weight.

[2]Mean ± S.E.

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

recognized at autopsy. Spleens from these animals were heavier than those from Group VIII when compared on an organ weight basis ($P < 0.01$) or as percentages of the body weights.

Kidney weights were similar for Groups VII (controls) and VIII. Kidneys from the magnesium-deficient animals (Group IX) were significantly heavier ($P < 0.01$) than those from either of the other groups on an organ weight and on a body-weight basis. The surfaces of the kidneys from Group IX were rough and pitted; kidneys were pale, and white deposits, probably calcium salts, were abundant and easily visible to the naked eye.

Livers from Group VIII (manganese-free) were smaller than control livers, but the difference was not significant. On a body-weight basis, livers from these animals were slightly larger than control livers. Magnesium-deficient rats had significantly smaller ($P < 0.01$) livers than the other 2 groups, but they were the same on a body-weight basis.

Total testes weight was the same for all groups, but control testes were significantly smaller than those from Group VIII ($P < 0.05$) or from Group IX ($P < 0.01$) when organ weights were expressed as percentages of the body weights. On that basis, the testes from Group IX were also significantly heavier ($P < 0.01$) than those from Group VIII. It was not uncommon for 1 testicle to be much smaller than the other.

Femur weights, measurements, and mineral concentrations

Femurs were cleaned as completely as possible by scraping away adhering tissue at the time of autopsy. Values for femur weights refer to the wet-weights of the cleaned bones (Table 15a). Control femur were

Table 15a. Femur weights, measurements; manganese and magnesium concentrations at autopsy in experiment 3

Group	Diet	Femur wt.		Femur diameters at positions indicated in Fig. 1				Femur length	Ash wt.	Mn/ Femur Ash	Femur Mn/ AWT	Mg/ Femur Ash	Femur Mg/ AWT
		g	% AWT[1]	D_1 mm	D_2 mm	D_3 mm	D_4 mm	mm	mg	ppm	ppm	ppm	ppm
VII	Control	0.80 ± 0.02[2,b]*	0.33 ± 0.01[b]	5.39 ± 0.09[b]	4.13 ± 0.05[b]	3.07 ± 0.04[b]	6.77 ± 0.09[b]	32.3 ± 0.4[b]	230.5 ± 3.9[b]	14.9 ± 0.8[b]	0.014 ± 0.005[b]	7429 ± 90[b]	6.9 ± 0.1[b]
VIII	Mn-free	0.75 ± 0.02[b]	0.35 ± 0.01[b]	5.20 ± 0.13[b]	4.16 ± 0.09[b]	3.08 ± 0.06[b]	6.72 ± 0.05[b]	31.1 ± 0.2[c]	207.7 ± 8.9[c]	15.6 ± 1.2[b]	0.015 ± 0.001[b]	7823 ± 101[b]	7.5 ± 0.2[b]
IX	Mg-deficient	0.66 ± 0.03[c]	0.39 ± 0.02[c]	4.65 ± 0.18[c]	3.96 ± 0.12[b]	3.11 ± 0.07[b]	6.53 ± 0.16[b]	28.1 ± 0.3[d]	168.6 ± 10.1[d]	18.4 ± 1.8[b]	0.018 ± 0.001[c]	3586 ± 1085[c]	3.5 ± 1.1[c]

[1] AWT = Autopsy weight.

[2] Mean ± S.E.

*Statistical analysis: means with same superscripts in a column are not different ($P > 0.05$).

heavier than those of Group IX on either a total femur-weight or body-weight basis ($P < 0.01$). They were significantly longer ($P < 0.01$) than those from Groups VIII and IX. Femurs from Group IX were also significantly shorter and lighter ($P < 0.01$) than those from Group VIII. Diameter at position D_1 (Figure 1) was significantly less ($P < 0.01$) for magnesium-deficient animals as compared to the other groups. Control femur ash weight was significantly heavier than that of Group VIII ($P < 0.05$) or Group IX ($P < 0.01$). Femur ash from magnesium-deficient animals also weighed less ($P < 0.01$) than the ash from Group VIII. Femur weight was positively correlated ($P < 0.01$) with femur length and both parameters were positively correlated ($P < 0.01$) with the ash weight. Magnesium deprivation, therefore, had a great effect on bone growth, affecting length, diameter, weight, and ash content. Manganese deficiency reduced length and ash content but to a lesser degree.

Manganese concentration in the femur-ash was not different among groups, but femurs from magnesium-deficient animals contained significantly more manganese ($P < 0.01$) in relation to body-weight. Therefore, there was no evidence of depletion of bone manganese when the deficient diet was fed.

Magnesium-deprivation reduced ($P < 0.01$) magnesium concentration in the femur-ash. The decrease was still significant ($P < 0.01$) when femur magnesium was expressed in terms of body weight. Thus, not only were animals smaller, but the concentration of magnesium in ash was also low.

Length of the bone was positively correlated with femur magnesium content ($P < 0.01$), while femur weight was positively correlated with

both femur-magnesium and femur-manganese ($P < 0.01$).

Length-diameter and diameter-diameter ratios at the positions indicated in Figure 1 were calculated to determine whether manganese and magnesium status affected relative dimensions of bones (Table 15b). Magnesium deficiency reduced ($P < 0.01$) bone length relative to bone diameter at positions D_2, D_3, and D_4 when compared to control and manganese-deficient animals. Manganese deficiency reduced ($P < 0.05$) bone length relative to bone diameter only at position D_4 when compared to the control group.

In most cases, magnesium deficiency also reduced ($P < 0.01$) bone diameter at position D_4 relative to D_1 and D_3, at D_1 relative to D_2 and D_3, and at D_2 relative to D_3 when compared to controls and manganese-deficient animals. The sum of the bone diameters taken at all positions was also less ($P < 0.05$) for Group IX than for control animals.

Manganese deficiency did not change relative diameters of femurs when compared with controls.

Splenic nucleic acids

RNA and DNA determinations were made on spleen homogenates (Table 16). Magnesium-deficient animals had greater total RNA than controls ($P < 0.05$) or Group VIII ($P < 0.01$). RNA concentration in spleen tissue was greatest in the magnesium-deficient animals; the difference approached significance when Group IX was compared with controls.

Total DNA was significantly less in spleens from manganese-deficient animals when compared with controls ($P < 0.05$) or with Group IX ($P < 0.01$).

Table 15b. Ratios of femur length (FL) to bone diameter (D) and ratios of bone diameters to each other (D/D) at the positions indicated in Figure 1, experiment 3

Group	Diet	FL/D_1 mm/mm	FL/D_2 mm/mm	FL/D_3 mm/mm	FL/D_4 mm/mm	D_4/D_1 mm/mm	D_4/D_2 mm/mm	D_4/D_3 mm/mm	D_1/D_2 mm/mm	D_1/D_3 mm/mm	D_2/D_3 mm/mm	$D_{1+2+3+4}$ mm
VII	Control	6.0^{a*}	7.8^{a}	10.5^{a}	4.8^{a}	1.26^{a}	1.64^{a}	2.21^{a}	1.31^{a}	1.76^{a}	1.35^{a}	19.36^{a}
VIII	Mn-free	6.0^{a}	7.5^{a}	10.1^{a}	4.6^{b}	1.30^{a}	1.62^{a}	$2.18^{a,b}$	$1.25^{a,b}$	1.68^{a}	1.35^{a}	$19.15^{a,b}$
IX	Mg-deficient	6.1^{a}	7.1^{b}	9.1^{b}	4.3^{c}	1.41^{b}	1.65^{a}	2.10^{b}	1.18^{b}	1.49^{b}	1.27^{b}	18.26^{b}

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

Table 16. Mean nucleic acid content of spleens in experiment 3

Group	Diet	Total RNA mg	mg RNA/ g spleen	Total DNA mg	mg DNA/ g spleen	RNA/ DNA
VII	Control	2.78 ± 0.17[1,b*]	4.58 ± 0.21[b]	10.70 ± 0.67[b]	17.52 ± 0.70[b]	0.26 ± 0.01[b]
VIII	Mn-free	2.37 ± 0.17[b]	4.88 ± 0.35[b]	8.66 ± 0.59[c]	17.56 ± 0.74[b]	0.28 ± 0.01[b]
IX	Mg-deficient	3.49 ± 0.26[c]	5.42 ± 0.46[b]	12.28 ± 0.88[b]	19.05 ± 1.36[b]	0.28 ± 0.01[b]

[1]Mean ± S.E.

*Statistical analysis: means with the same superscripts in a column are not different ($P > 0.05$).

The differences were not apparent when comparisons were made on the basis of concentration in splenic tissue, however, so the decrease was due to the smaller total size of the spleen in these animals.

RNA concentrations in cells were similar among all groups, as indicated by the RNA-DNA ratio. Both mineral deficiencies tended to increase RNA concentration per cell and possibly to reduce cell-size relative to that of controls, but the differences were not statistically significant.

Antibody titers and gamma globulin fractions

Agglutinin and hemolysin titers were determined 5 and 9 days following immunization with sheep red blood cells (SRBC). These data are summarized in Table 17. Magnesium deficiency lowered day 5 agglutinin and hemolysin titers ($P < 0.01$) in comparison to Groups VII and VIII. Control values (Group VII) for day 9 agglutinin titers were significantly higher ($P < 0.05$) than corresponding values for groups deficient in either mineral. There were no differences among groups for day 9 hemolysin values, although mean titer for Group IX was considerably lower than for the other groups. Magnesium deficiency seemed to affect the time at which maximum agglutinin and hemolysin levels were reached, because highest titers for Group IX were observed on day 9, while highest titers for Groups VII and VIII occurred on day 5 for both antibodies. All serum antibody levels were positively correlated ($P < 0.01$) with femur-magnesium concentrations, but not with manganese content.

Table 17. Mean serum antibody titers 5 and 9 days following immunization and serum gamma globulin fractionation data in experiment 3

Group	Diet	Agglutinin Day 5	Agglutinin Day 9	Hemolysin Day 5	Hemolysin Day 9	Protein G.G. ext.[1,2]	Total protein[1] (7S+19S + other)	Protein 19S	Protein 19S	Protein 7S	Protein 7S
		log titer		log titer		mg/100 ml	mg/100 ml	mg/100 ml	%T[3]	mg/100 ml	%T
VII	Control	1.78 ± 0.16[4,b]*	1.63 ± 0.13[b]	2.95 ± 0.32[b]	2.40 ± 0.18[b]	21.44 ± 2.84[b]	52.47 ± 4.93[b,c]	5.82 ± 0.36[b]	12.11 ± 1.26[b]	36.42 ± 4.85[b,c]	67.67 ± 3.05[b,c]
VIII	Mn-free	1.66 ± 0.23[b]	1.21 ± 0.19[c]	2.86 ± 0.38[b]	2.51 ± 0.33[b]	21.60 ± 3.34[b]	40.20 ± 5.72[b]	3.63 ± 0.46[c]	9.71 ± 1.21[b,c]	25.61 ± 4.10[b]	63.00 ± 2.92[b]
IX	Mg-deficient	0.80 ± 0.21[c]	1.06 ± 0.13[c]	1.30 ± 0.10[c]	1.93 ± 0.29[b]	55.44 ± 9.99[c]	61.37 ± 6.65[c]	3.69 ± 0.54[c]	6.71 ± 1.10[c]	45.99 ± 5.26[c]	74.29 ± 1.39[c]

[1]Total protein values for 7S + 19S + Other were determined on solutions 2.5X more concentrated than solutions on which G.G. ext. protein values were measured.

[2]G.G. ext. = Gamma globulin extract.

[3]%T = % of total protein (7S + 19S + Other).

[4]Mean ± S.E.

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

Protein concentrations of the serum gamma globulin extract before separation and of its 19S and 7S sub-fractions were determined by ultraviolet spectrophotometry and expressed as mg protein/100 ml solution.

The protein concentrations of the sub-fractions were added to that of an unidentified fraction to give total protein after separation. The sub-fractions were also expressed as percentages of this total protein value. Values for protein concentrations of the gamma globulin extracts and total protein are not directly comparable because total protein values were determined on solutions 2.5 times more concentrated.

Protein levels in the gamma globulin extracts were significantly higher ($P < 0.01$) from Group IX serum than from either of the other groups. Total protein concentration obtained by adding sub-fraction values was also higher in the magnesium-deficient group (IX), although the increase was significant ($P < 0.05$) only over Group VIII. Protein concentrations for the 19S sub-fractions from Groups VIII and IX were lower ($P < 0.05$) than those from controls (Group VII). The same trend was seen when 19S protein was expressed as a percentage of the total for fractions measured, but the decrease was significant ($P < 0.01$) only when magnesium-deficient animals were compared to controls.

The 7S fraction tended to be higher for the magnesium-deficient group, when values were expressed either as mg/100 ml or as percentages of the total protein. In both cases, however, the differences were significant ($P < 0.01$) only between Groups VIII and IX.

Protein in the gamma globulin extracts was negatively correlated ($P < 0.01$) with both values for the 19S sub-fraction and positively

correlated ($P < 0.01$) with values for the 7S sub-fraction and total protein.

Experiment 4

Deficiency of magnesium in the weanling rats of experiment 3 had produced dramatic reductions in serum antibody titers. The primary purpose of experiment 4 was to determine the effects of magnesium deficiency on the immune response of older rats. It was assumed that these older slow-growing animals had greater magnesium reserves than the weanlings, so they were fed a "magnesium-free" diet, containing 1 mg magnesium per 100 gm diet (2.5% of NRC recommendation for magnesium), during the entire experimental period (Table 4b). Controls received 48 mg magnesium per 100 gm diet (120% of NRC recommendation for magnesium).

Body weight change, food intake, and food-efficiency

Two-hundred-gram, male rats were obtained from the I.S.U. stock colony where they had been fed "stock" diet (Patten, 1970). They were placed immediately on either the control diet (Group X) or a diet containing no added magnesium (Group XI) for 38 days (Table 4a, 4b). Mean body-weight changes, food intakes and food efficiencies for both groups are summarized in Table 18. Symptoms of magnesium deficiency reported for the magnesium-deficient animals of experiment 3 were observed in the magnesium-deficient animals of Group XI in this experiment. Animals fed the "magnesium-free" diet gained only ½ as much weight during the 38-day period as controls. This difference was highly significant ($P < 0.01$). Food intake was also lower ($P < 0.01$)

Table 18. Mean body weight changes, food intakes, and food efficiencies in experiment 4

Group	Diet	$BWCh_T$ g [1,2]	FI_T g [3]	FER_T [4]	FER_1	FER_2	FER_3	FER_4	FER_5	FER_6
X	Control	154 ± 4 [5,f*]	627 ± 14[f]	0.25 ± 0.01[f]	0.18 ± 0.03[f]	0.36 ± 0.01[f]	0.27 ± 0.02[f]	0.21 ± 0.02[f]	0.22 ± 0.01[f]	0.21 ± 0.03[f]
XI	Mg-free	38 ± 5[g]	475 ± 21[g]	0.08 ± 0.01[g]	0.13 ± 0.01[f]	0.13 ± 0.04[g]	0.11 ± 0.02[g]	0.03 ± 0.02[g]	0.03 ± 0.03[g]	-0.05 ± 0.07[6,g]

[1] BWCh = Body weight change.

[2] T = Total.

[3] FI = Food intake.

[4] FER = Food efficiency ratio (wt. change (g)/food intake (g)); numbered subscripts refer to respective weekly periods.

[5] Mean ± S.E.

[6] Rats lost weight.

* Statistical analysis: means with the same superscripts in a column are not different ($P > 0.05$).

but could account for only part of the difference in weight gain, because the efficiency of food utilization was also less ($P < 0.01$). Differences in food-efficiency were highly significant ($P < 0.01$) after the first week, one indication of the speed with which magnesium deficiency was produced in these rats.

Autopsy and organ weights

Autopsy weights (Table 19) reflected the differences in weight gain for the 2 groups and were greater ($P < 0.01$) for control animals. Splenomegaly was quite apparent at autopsy. Spleens and testes were heavier in magnesium-deficient animals when compared with controls on a total organ weight basis ($P < 0.01$ and $P < 0.05$, respectively) or when tissue weights were expressed as percentages of the autopsy weights ($P < 0.01$). Spleen weights were negatively correlated with femur-magnesium and femur-manganese concentrations ($P < 0.01$).

Total kidney weights (Table 19) did not differ, but kidneys from magnesium-deficient animals (Group XI) were heavier ($P < 0.01$) when weights were expressed as percentages of the body weights. The surfaces of the kidneys from Group XI were rough and pitted with visible white deposits of what were probably calcium salts.

Livers (Table 19) from Group XI were smaller ($P < 0.01$) than those of controls but were not smaller in proportion to body size.

Femur weights, measurements, and mineral concentrations

Adhering tissue was removed as completely as possible from the femurs at the time of autopsy. Femur weights in Table 20a refer to the wet weights of the bones and did not differ between the groups. When femur

Table 19. Mean body and organ weights at autopsy in experiment 4

Group	Diet	Autopsy wt. g	Spleen wt. g	Spleen wt. % AWT[1]	Kidney wt. g	Kidney wt. % AWT	Liver wt. g	Liver wt. % AWT	Testes wt. g	Testes wt. % AWT
X	Control	348 ± 4[2,c*]	0.75 ± 0.03[c]	0.21 ± 0.01[c]	2.97 ± 0.06[c]	0.85 ± 0.02[c]	12.80 ± 0.29[c]	3.68 ± 0.07[c]	3.37 ± 0.11[c]	0.97 ± 0.04[c]
XI	Mg-free	230 ± 5[d]	1.09 ± 0.07[d]	0.48 ± 0.03[d]	2.92 ± 0.12[c]	1.27 ± 0.07[d]	8.67 ± 0.41[d]	3.75 ± 0.12[c]	3.77 ± 0.18[d]	1.63 ± 0.06[d]

[1] AWT = Autopsy weight.

[2] Mean ± S.E.

*Statistical analysis: means with the same superscripts in a column are not different ($P > 0.05$).

Table 20a. Femur weights, measurements; manganese and magnesium concentrations at autopsy in experiment 4

Group	Diet	Femur wt.		Femur diameters at positions indicated in Fig. 1				Femur length	Ash wt.	Mn/ Femur Ash	Femur Mn/ AWT	Mg/ Femur Ash	Femur Mg/ AWT
				D_1	D_2	D_3	D_4						
		g	% AWT[1]	mm	mm	mm	mm	mm	mg	ppm	ppm	ppm	ppm
X	Control	1.08 ± 0.03[2,b]*	0.31 ± 0.01[b]	5.94 ± 0.11[b]	4.63 ± 0.12[b]	3.51 ± 0.07[b]	6.97 ± 0.20[b]	36.6 ± 0.4[b]	338.8 ± 8.1[b]	12.1 ± 0.6[b]	0.012 ± 0.000[b]	6713 ± 89[b]	6.5 ± 0.2[b]
XI	Mg-free	1.04 ± 0.04[b]	0.45 ± 0.02[c]	5.55 ± 0.09[c]	4.55 ± 0.18[b]	3.47 ± 0.06[b]	7.00 ± 0.12[b]	33.1 ± 0.3[c]	263.5 ± 4.9[c]	12.8 ± 0.5[b]	0.015 ± 0.000[c]	3537 ± 971[c]	4.1 ± 1.1[c]

[1]AWT = Autopsy weight.

[2]Mean ± S.E.

*Statistical analysis: means with same superscripts in a column are not different ($P > 0.05$).

weights were expressed as percentages of body weights, however, femurs from magnesium-deficient rats (Group XI) were heavier. These data indicate that magnesium deficiency affected body weight significantly more than femur weight. Femurs from the magnesium-deficient animals (Group XI) were shorter ($P < 0.01$) than those from controls. Diameter at position D_1 (Figure 1) was greater ($P < 0.05$) for controls. Magnesium deficiency did not affect bone diameter at the other positions measured, however. Control femur ash weight was heavier ($P < 0.01$) than femur ash from Group XI. Femur weight tended to be positively correlated ($P < 0.07$) with length, and these parameters were correlated (weight, $P < 0.07$ and length, $P < 0.01$) with femur-ash weight. Femur length was positively correlated with femur magnesium and manganese concentrations in ash ($P < 0.01$), while femur weight tended to be positively correlated with femur magnesium ($P < 0.06$). Femur manganese was positively correlated with femur magnesium ($P < 0.01$), so it was expected that femur length was positively correlated with femur content of both minerals.

Manganese concentration (Table 20a) of femur ash was not different between the 2 groups, but femurs from magnesium-deficient animals contained more manganese ($P < 0.01$) on a body-weight basis.

Magnesium deprivation caused decreases ($P < 0.01$) of the order of 50% in magnesium concentration in the femur ash (Table 20a). The decrease remained apparent ($P < 0.05$) when femur magnesium was expressed in terms of body weight. The smaller size of the animals, therefore, did not compensate for the decrease in total femur magnesium.

Reductions in femur length relative to bone diameter at positions D_3 and D_4 (Figure 1) were associated with magnesium deficiency ($P < 0.01$ and $P < 0.05$, respectively); there was a tendency for a similar reduction at position D_2 ($P < 0.09$) (Table 20b). This data and the figures on femur diameters and length from Table 20a show that magnesium deficiency reduced bone length more than bone diameter.

Bone-diameter ratios were not affected by dietary magnesium. The sums of the bone diameters were similar, further supporting the observation that magnesium-deficiency affected bone length more than bone diameter.

Splenic nucleic acids

Spleen homogenates were analyzed for RNA and DNA content (Table 21). Total values for both nucleic acids were greater ($P < 0.01$) in spleens from magnesium-deficient animals. The effect related directly to the larger total size of the spleen in magnesium deficiency, because the groups were not different when values were compared on a tissue concentration basis. RNA concentrations per cell were also similar among all groups, as indicated by the RNA-DNA ratio.

Antibody titers and gamma globulin fractions

Agglutinin and hemolysin titers were determined 5 and 9 days following immunization with sheep red blood cells. Titers of both antibodies were lower ($P < 0.01$) in sera from magnesium-deficient animals on day 5 and day 9 (Table 22). The times at which maximum agglutinin and hemolysin levels were reached apparently were similar in both groups; maximum titers occurred on day 9 for agglutinin and on day

Table 20b. Ratios of femur length (FL) to bone diameters (D) and ratios of bone diameters to each other (D/D) at the positions indicated in Figure 1, experiment 4

Group	Diet	FL/D_1 mm/mm	FL/D_2 mm/mm	FL/D_3 mm/mm	FL/D_4 mm/mm	D_4/D_1 mm/mm	D_4/D_2 mm/mm	D_4/D_3 mm/mm	D_1/D_2 mm/mm	D_1/D_3 mm/mm	D_2/D_3 mm/mm	$D_{1+2+3+4}$ mm
X	Control	6.2^{a*}	7.9^{a}	10.5^{a}	5.3^{a}	1.17^{a}	1.51^{a}	1.99^{a}	1.29^{a}	1.70^{a}	1.33^{a}	21.04^{a}
XI	Mg-free	6.0^{a}	7.3^{a}	9.6^{b}	4.7^{b}	1.26^{a}	1.55^{a}	2.02^{a}	1.23^{a}	1.60^{a}	1.31^{a}	20.57^{a}

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

Table 21. Mean nucleic acid content of spleens in experiment 4

Group	Diet	Total RNA mg	mg RNA/ g spleen	Total DNA mg	mg DNA/ g spleen	RNA/ DNA
X	Control	3.81 ± 0.21[1,a*]	5.09 ± 0.16[a]	12.69 ± 0.62[a]	17.03 ± 0.52[a]	0.30 ± 0.01[a]
XI	Mg-free	5.73 ± 0.60[b]	5.19 ± 0.33[a]	18.01 ± 1.12[b]	16.56 ± 0.74[a]	0.32 ± 0.03[a]

[1]Mean ± S.E.

*Statistical analysis: means with the same superscripts in a column are not different ($P > 0.05$).

5 for hemolysin. The greater percentage decrease in hemolysin titers from day 5 to day 9 for control animals, may reflect differences in either turnover rate or in the exact time at which peak titer was achieved. Nevertheless, even on day 9, log titers were almost 2 times as great as those for magnesium-deficient animals. Agglutinin and hemolysin values were correlated ($P < 0.01$ and $P < 0.05$, respectively) with femur-magnesium concentrations on days 5 and 9.

Protein concentrations of gamma globulin extracts and of the 19S and 7S sub-fractions were expressed as mg protein per 100 ml solution (Table 22). The protein concentrations of the sub-fractions were added with that of other fractions to give total protein. The sub-fractions were also expressed as percentages of this total protein value. Values for protein concentrations of the gamma globulin extracts and total protein are not directly comparable because total protein values were determined on solutions 2.5 times more concentrated.

Protein levels in the gamma globulin extracts were similar for the two groups. Total protein concentration obtained by adding sub-fraction values was lower in magnesium-deficient animals ($P < 0.05$). Even though the values for the gamma globulin extract and total protein from the sub-fractions are not the same, changes in the two induced by experimental treatment should be parallel; this was not true in experiment 4.

Protein concentrations for the 19S fractions (mg/100 ml) were similar. When 19S protein was expressed as a percentage of the sub-fraction total, values were higher ($P < 0.05$) for magnesium-deficient

Table 22. Mean serum antibody titers 5 and 9 days following immunization and serum gamma globulin fractionation data in experiment 4

Group	Diet	Agglutinin Day 5	Agglutinin Day 9	Hemolysin Day 5	Hemolysin Day 9	Protein G.G. ext.[1,2]	Total protein[1] (7S+19S + Other)	Protein 19S	Protein 19S	Protein 7S	Protein 7S
		log titer		log titer		mg/100 ml	mg/100 ml	mg/ 100 ml	%T[3]	mg/100 ml	%T
X	Control	2.06 ± 0.21[4,b]*	3.06 ± 0.19[b]	2.18 ± 0.20[b]	1.68 ± 0.18[b]	28.53 ± 4.28[b]	61.93 ± 5.05[b]	4.46 ± 0.75[b]	7.10 ± 0.72[b]	48.39 ± 4.51[b]	79.10 ± 1.10[b]
XI	Mg-free	0.61 ± 0.09[c]	0.76 ± 0.15[c]	0.93 ± 0.17[c]	0.89 ± 0.10[c]	30.48 ± 7.57[b]	45.57 ± 2.39[c]	4.62 ± 0.46[b]	10.33 ± 1.33[c]	32.35 ± 2.34[c]	70.83 ± 2.02[c]

[1]Total protein values for 7S + 19S + Other were determined on solutions 2.5X more concentrated than solutions on which G.G. ext. protein values were measured.

[2]G.G. ext. = Gamma globulin extract.

[3]%T = % of total protein (7S + 19S + Other).

[4]Mean ± S.E.

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

rats than for control rats.

Protein concentrations for the 7S (mg/100 ml) were higher ($P < 0.05$) in control rats. The same effect was observed when values were expressed as percentages of the added fraction values ($P < 0.01$).

Agglutinin values tended to be negatively correlated with 19S protein values expressed as percentages of fraction totals ($P < 0.07$ and $P < 0.04$ for days 5 and 9, respectively). Hemolysin values were also negatively correlated with the same values ($P < 0.03$ and $P < 0.08$ for days 5 and 9, respectively). Agglutinin values on day 9 were positively correlated with the protein values of the 7S fraction, expressed as percentages of total protein ($P < 0.05$), but hemolysin was not related to 7S concentration.

Experiment 5

Experiment 1 had failed to demonstrate clear effects of level and ratio of manganese fed on the immune response of rats. The purposes of experiment 5 were similar to those of experiment 1. Protein concentration was doubled, and dextrose was substituted for cornstarch. Manganese and other minerals which were varied (Table 1, Group XV) were increased to 5 times the NRC requirement to increase the possibility of producing differences among groups.

Body weight change, food intake, and food-efficiency

Weanling rats from the I.S.U. stock colony were fed diets as indicated in Table 1. Manganese contents of the control and "manganese-free" diets were 4.7 and 0.1 mg per 100 gm diet, respectively, by

analysis and provided 94% and 2% of the NRC requirement (Table 4b). Other diets were assumed to contain 5 times the control value for manganese.

Mean body-weight changes, food intakes and food efficiencies for all groups are given in Table 23. Analysis of variance revealed no differences among group means for any of the 3 parameters. When values for least-significant difference were used for comparing groups, Group XIV, fed 5 times adequate manganese, had a greater total food efficiency ratio compared to the other groups ($P < 0.05$ for XII and XV; $P < 0.01$ for XIII, "manganese-free"). Body weight changes and food intakes among groups were similar, however, so the greater food efficiency indicated for Group XIV may have been an artifact.

Autopsy and organ weights

Analysis of variance revealed no differences among groups for body weights or for tissue weights at autopsy (Table 24). The one exception was a greater total liver weight ($P < 0.05$) for Group XIV, fed 5 times adequate manganese. The difference was removed when liver weights were expressed as percentages of the autopsy weights.

Femur weights, measurements, and mineral concentrations

No differences in femur weights or in femur lengths or diameters were demonstrated (Table 25a). Ash weights and femur-manganese concentrations were also similar. Group XV, fed 5 times adequate levels of manganese, magnesium, iron, copper, and zinc had higher levels of femur magnesium ($P < 0.01$) than the other groups. This is in agreement with

Table 23. Mean body weight changes, food intakes, and food efficiencies in experiment 5

Group	Diet	$BWCh_T$[1,2] g	FI_T[3] g	FER_T[4]	FER_1	FER_2	FER_3	FER_4	FER_5	FER_6	FER_7	FER_8
XII	Control	224 ± 7[5,g*]	662 ± 11[g]	0.34 ± 0.01[g]	0.33 ± 0.02[g]	0.47 ± 0.01[g]	0.53 ± 0.03[g]	0.43 ± 0.03[g]	0.33 ± 0.02[g]	0.29 ± 0.02[g]	0.20 ± 0.02[g]	0.29 ± 0.04[g]
XIII	Mn-free	209 ± 11[g]	626 ± 32[g]	0.33 ± 0.01[g]	0.31 ± 0.03[g]	0.49 ± 0.02[g,h]	0.42 ± 0.05[h]	0.45 ± 0.03[g]	0.31 ± 0.03[g]	0.30 ± 0.02[g]	0.24 ± 0.04[g,h]	0.22 ± 0.08[g]
XIV	5 x Mn	229 ± 9[g]	637 ± 20[g]	0.36 ± 0.01[h]	0.41 ± 0.03[h]	0.53 ± 0.01[h]	0.51 ± 0.04[g,h]	0.35 ± 0.03[h]	0.35 ± 0.02[g]	0.30 ± 0.03[g]	0.29 ± 0.01[h]	0.26 ± 0.02[g]
XV	5 x Complex[6]	205 ± 11[g]	596 ± 29[g]	0.34 ± 0.01[g]	0.28 ± 0.07[g]	0.49 ± 0.02[g,h]	0.50 ± 0.02[g,h]	0.45 ± 0.02[g]	0.34 ± 0.02[g]	0.30 ± 0.02[g]	0.28 ± 0.03[h]	0.18 ± 0.03[g]

[1] BWCh = Body weight change.

[2] T = Total.

[3] FI = Food intake.

[4] FER = Food efficiency ratio (wt. change (g)/food intake (g)); numbered subscripts refer to respective weekly periods.

[5] Mean ± S.E.

[6] 5 x Mn, Mg, Fe, Cu, Zn.

*Statistical analysis: means with the same superscripts in a column are not different ($P > 0.05$).

Table 24. Mean body and organ weights at autopsy in experiment 5

Group	Diet	Autopsy wt. g	Spleen wt. mg	Spleen wt. % AWT[1]	Kidney wt. g	Kidney wt. % AWT	Liver wt. g	Liver wt. % AWT	Testes wt. g	Testes wt. % AWT
XII	Control	274 ± 8[2,c*]	671.2 ± 44.0[c]	0.24 ± 0.01[c]	2.36 ± 0.08[c]	0.86 ± 0.02[c]	10.14 ± 0.48[c]	3.71 ± 0.15[c]	3.31 ± 0.07[c]	1.22 ± 0.06[c]
XIII	Mn-free	259 ± 11[c]	629.5 ± 34.3[c]	0.24 ± 0.01[c]	2.32 ± 0.11[c]	0.90 ± 0.04[c]	9.04 ± 0.43[c]	3.49 ± 0.09[c]	3.16 ± 0.13[c]	1.22 ± 0.04[c]
XIV	5 x Mn	278 ± 9[c]	671.9 ± 26.0[c]	0.24 ± 0.01[c]	2.52 ± 0.14[c]	0.90 ± 0.03[c]	10.53 ± 0.31[d]	3.79 ± 0.09[c]	3.40 ± 0.08[c]	1.23 ± 0.05[c]
XV	5 x Complex[3]	254 ± 11[c]	602.9 ± 40.2[c]	0.24 ± 0.01[c]	2.26 ± 0.09[c]	0.89 ± 0.01[c]	9.56 ± 0.49[c]	3.77 ± 0.15[c]	3.12 ± 0.19[c]	1.23 ± 0.07[c]

[1] AWT = Autopsy weight.

[2] Mean ± S.E.

[3] 5 x Mn, Mg, Fe, Cu, Zn.

*Statistical analysis: means with the same superscript in a column are not different ($P > 0.05$).

Table 25a. Femur weights, measurements; manganese and magnesium concentrations at autopsy in experiment 5

Group	Diet	Femur wt.		Femur diameters at positions indicated in Fig. 1				Femur length	Ash wt.	Mn/ Femur Ash	Femur Mn/ AWT	Mg/ Femur Ash	Femur Mg/ AWT
				D_1	D_2	D_3	D_4						
		mg	% AWT[1]	mm	mm	mm	mm	mm	mg	ppm	ppm	ppm	ppm
XII	Control	880.3 ± 21.7[2,b*]	0.32 ± 0.01[b]	5.58 ± 0.04[b]	4.24 ± 0.05[b]	3.21 ± 0.05[b]	6.75 ± 0.08[b,c]	33.4 ± 0.3[b]	259.8 ± 9.3[b]	12.1 ± 0.5[b]	0.011 ± 0.000[b]	6958 ± 265[b]	6.6 ± 0.3[b]
XIII	Mn-free	858.2 ± 26.1[b]	0.33 ± 0.01[b]	5.42 ± 0.11[b]	4.33 ± 0.08[b]	3.14 ± 0.04[b]	6.82 ± 0.04[b]	32.8 ± 0.5[b]	257.6 ± 9.4[b]	11.6 ± 0.6[b]	0.012 ± 0.000[b]	6772 ± 186[b]	6.7 ± 0.6[b]
XIV	5 x Mn	886.7 ± 26.5[b]	0.32 ± 0.01[b]	5.73 ± 0.15[b]	4.40 ± 0.12[b]	3.25 ± 0.04[b]	6.70 ± 0.05[b,c]	33.5 ± 0.4[b]	259.7 ± 8.5[b]	13.2 ± 0.8[b]	0.012 ± 0.000[b]	6942 ± 60[b]	6.5 ± 0.1[b]
XV	5 x Complex[3]	823.2 ± 21.4[b]	0.33 ± 0.01[b]	5.61 ± 0.14[b]	4.36 ± 0.08[b]	3.24 ± 0.05[b]	6.62 ± 0.08[c]	32.8 ± 0.4[b]	238.9 ± 9.4[b]	20.4 ± 7.9[b]	0.020 ± 0.010[b]	8590 ± 288[c]	8.1 ± 0.3[c]

[1] AWT = Autopsy weight.

[2] Mean ± S.E.

[3] 5 x Mn, Mg, Fe, Cu, Zn.

*Statistical analysis: means with same superscripts in a column are not different ($P > 0.05$).

Table 25b. Ratios of femur length (FL) to bone diameter (D) and ratios of bone diameters to each other (D/D) at the positions indicated in Figure 1, experiment 5

Group	Diet	FL/D_1 mm/mm	FL/D_2 mm/mm	FL/D_3 mm/mm	FL/D_4 mm/mm	D_{4/D_1} mm/mm	D_{4/D_2} mm/mm	D_{4/D_3} mm/mm	D_{1/D_2} mm/mm	D_{1/D_3} mm/mm	D_{2/D_3} mm/mm	$D_{1+2+3+4}$ mm
XII	Control	6.0^{a*}	7.9^{a}	10.4^{a}	$4.9^{a,b}$	$1.21^{a,b}$	1.59^{a}	2.10^{a}	1.32^{a}	1.74^{a}	1.32^{a}	19.79^{a}
XIII	Mn-free	6.1^{a}	$7.6^{a,b}$	10.5^{a}	4.8^{b}	1.26^{a}	1.58^{a}	2.18^{b}	1.25^{b}	1.73^{a}	1.38^{b}	19.70^{a}
XIV	5 x Mn	5.9^{a}	$7.6^{a,b}$	10.3^{a}	5.0^{a}	1.17^{b}	1.53^{a}	2.07^{a}	$1.30^{a,b}$	1.76^{a}	$1.35^{a,b}$	20.08^{a}
XV	5 x Complex[1]	5.9^{a}	7.5^{b}	10.1^{a}	5.0^{a}	1.18^{b}	1.83^{b}	2.04^{a}	1.29^{a}	1.73^{a}	$1.35^{a,b}$	19.84^{a}

[1] 5 x Mn, Mg, Fe, Cu, Zn.

*Statistical analysis: means with same superscripts in a column are not different ($P > 0.05$).

the observations from experiments 3 and 4 that femur magnesium varied directly with the magnesium content of the diets fed.

Length-diameter and diameter-diameter ratios at the positions indicated in Figure 1 were calculated to determine whether the dietary mineral variations changed the relative dimensions of bones (Table 25b). Analysis of variance revealed differences among groups in the D_4/D_1 and D_4/D_3 ratios. The group fed the "manganese-free" diet tended to be smaller in diameter at D_1 and D_3 and larger in diameter at D_4 than the other groups (Table 25a), although the absolute differences were not significant. When expressed on a relative basis (D_4/D_1, D_4/D_3), however, the difference between this group and the other groups became significant ($P < 0.01$ for Groups XIV and XV and $P < 0.05$ for controls).

Splenic nucleic acids

RNA and DNA determinations were made on spleen homogenates (Table 26). Analysis of variance revealed no differences among groups for total RNA or DNA, in the concentration of either, or in the RNA-DNA ratio. Tests of least-significant difference showed that animals fed the "manganese-free" diet (Group XIII) had a higher concentration of splenic RNA ($P < 0.05$) than animals fed 5 times adequate manganese (Group XV). This difference was reflected in the relative RNA-DNA ratios between the groups since DNA values between the groups were similar; the "manganese-free" group tended to have a greater concentration of RNA in cells than other groups. Spleen cell size tended to be larger for Groups XIII and XIV as indicated by the somewhat lower values for DNA concentration.

Table 26. Mean nucleic acid content of spleens in experiment 5

Group	Diet	Total RNA mg	mg RNA/ g spleen	Total DNA mg	mg DNA/ g spleen	RNA/ DNA
XII	Control	3.54 ± 0.18[1,b*]	5.34 ± 0.20[b,c]	11.45 ± 0.67[b]	17.18 ± 0.54[b]	0.31 ± 0.01[b,c]
XIII	Mn-free	3.45 ± 0.19[b]	5.49 ± 0.15[b]	10.50 ± 0.46[b]	16.77 ± 0.51[b]	0.33 ± 0.01[b]
XIV	5 x Mn	3.37 ± 0.15[b]	5.01 ± 0.08[c]	11.33 ± 0.57[b]	16.88 ± 0.66[b]	0.30 ± 0.01[c]
XV	5 x Complex[2]	3.22 ± 0.16[b]	5.39 ± 0.11[b,c]	10.46 ± 0.68[b]	17.40 ± 0.56[b]	0.31 ± 0.01[b,c]

[1]Mean ± S.E.

[2]5 x Mn, Mg, Fe, Cu, Zn.

*Statistical analysis: means with same superscripts in a column are not different ($P > 0.05$).

Antibody titers

Agglutinin and hemolysin titers were determined 5 and 7 days following immunization with sheep red blood cells (Table 27). There were no differences among groups for days 5 and 7 agglutinin or day 7 hemolysin values. Analysis of variance indicated no differences among groups ($P < 0.09$) for day 5 hemolysin values, but the test of least-significant difference showed that feeding the mixture of minerals at 5 times adequate levels (Groups XV) reduced hemolysin titers relative to the controls.

Experiment 6

The purposes of experiment 6 were a) to investigate interrelationships in magnesium, manganese, tryptophan and vitamin B_6 metabolism in rats fed low-magnesium diets and the effects of these interrelationships on their immune responses and b) to determine whether the depression in antibody formation in magnesium-deficient rats of experiments 3 and 4 was due to inadequate magnesium or to the decrease in protein intake resulting from a lower food intake. To assess the effects of the dietary treatments on the various parameters measured, analysis of variance was performed. In addition, a factorial analysis of variance was performed on Groups XVI, XVII, XIX, XX, XXI, and XXIII to determine the effects of level of magnesium fed (¼ of NRC requirement versus 5¼ times the NRC requirement), the effects of 2 supplements (vitamin B_6 and B_6 + tryptophan) fed with the 2 levels of magnesium, and the interaction of magnesium with the supplements.

Table 27. Mean serum antibody titers 5 and 7 days following immunization in experiment 5

Group	Diet	Agglutinin log titer Day 5	Agglutinin log titer Day 7	Hemolysin log titer Day 5	Hemolysin log titer Day 7
XII	Control	2.90 ± 0.24[1,c*]	3.39 ± 0.09[c]	2.30 ± 0.10[c]	2.79 ± 0.13[c]
XIII	Mn-free	2.20 ± 0.25[c]	3.42 ± 0.09[c]	2.00 ± 0.08[c,d]	2.67 ± 0.14[c]
XIV	5 x Mn	2.45 ± 0.42[c]	3.37 ± 0.24[c]	2.03 ± 0.26[c,d]	2.74 ± 0.24[c]
XV	5 x Complex[2]	1.96 ± 0.41[c]	3.10 ± 0.11[c]	1.68 ± 0.20[d]	2.41 ± 0.09[c]

[1]Mean ± S.E.

[2]5 x Mn, Mg, Fe, Cu, Zn.

*Statistical analysis: means with same superscripts in a column are not different ($P > 0.05$).

Body weight change, food intake, and food-efficiency

Data for these parameters are presented in Table 28 for the C-period (time during which the rats reached 80±5 grams body weight), the E-period (35 days following the C-period), and the D-period (11 day period following the E-period, during which experimental diets were fed) (Table 1). Analysis of variance revealed no differences among groups for body weight changes, food intakes or food efficiencies during the C-period. This was expected, as all rats were fed the control diet until they reached 80±5 grams body weight. Group designations other than assignment to the 21.5% protein (XXIV) or 30% protein group (XXV) were made after the E-period, when the 80 rats which had been fed ¼-magnesium diets for 35 days were randomly assigned to experimental diets for the 11 day D-period. This fact is reflected in the body weight changes, food intakes and food efficiencies during the E-period. These values were similar for Groups XVI-XXIII, which were all fed the ¼-magnesium diet during this time. Values for each parameter were similar when comparing the 2 groups receiving adequate magnesium and different protein levels (Groups XXIV and XXV). Groups XXIV and XXV, receiving adequate magnesium, had greater weight gains, food intakes ($P < 0.01$), and food efficiencies ($P < 0.05$) than animals receiving inadequate magnesium, Groups XVI-XXIII. Food efficiency was higher for the control group than for the magnesium-deficient groups. Food efficiency was intermediate for the 21.5% protein group and was not different from that for magnesium-deficient animals or that for control animals.

Table 28. Mean body weight changes, food intakes, and food efficiencies in experiment 6

Group	Exp. diet	$BWCh_C$[1,2]	FI_C[3]	FER_C[4]	$BWCh_E$[5]	FI_E	FER_E
P > F within a column[6]		0.7702	0.5197	0.6258	0.0001	0.0001	0.0167
		g	g		g	g	
XVI	½ Mg	36 ± 2[7,e*]	81 ± 5[e,f]	0.44 ± 0.01[e]	130 ± 6[e]	472 ± 13[e]	0.27 ± 0.01[e]
XVII	5X B_6	34 ± 1[e]	75 ± 4[e,f]	0.45 ± 0.01[e]	134 ± 10[e]	450 ± 14[e]	0.30 ± 0.02[e,f]
XVIII	5X tryp	36 ± 2[e]	83 ± 6[e,f]	0.44 ± 0.02[e]	132 ± 7[e]	474 ± 20[e]	0.28 ± 0.01[e]
XIX	½ Mg + 5X B_6	34 ± 1[e]	71 ± 5[e]	0.51 ± 0.05[e]	142 ± 7[e]	471 ± 17[e]	0.30 ± 0.01[e,f]
XX	5X tryp + 5X B_6	37 ± 2[e]	78 ± 6[e,f]	0.48 ± 0.03[e]	123 ± 17[e]	445 ± 34[e]	0.27 ± 0.02[e]
XXI	½ Mg + 5X tryp + 5X B_6	38 ± 2[e]	78 ± 4[e,f]	0.49 ± 0.02[e]	131 ± 9[e]	469 ± 17[e]	0.28 ± 0.01[e]
XXII	[Mn = 5X Mg] + ½ Mg	36 ± 2[e]	76 ± 4[e,f]	0.49 ± 0.04[e]	138 ± 10[e]	465 ± 21[e]	0.29 ± 0.01[e]
XXIII	½ Mg	38 ± 1[e]	87 ± 6[f]	0.45 ± 0.04[e]	124 ± 11[e]	450 ± 19[e]	0.27 ± 0.02[e]
XXIV	21.5% protein	35 ± 2[e]	81 ± 6[e,f]	0.45 ± 0.02[e]	173 ± 3[f]	584 ± 17[f]	0.30 ± 0.01[e,f]
XXV	Control (30% protein)	36 ± 1[e]	74 ± 4[e,f]	0.49 ± 0.02[e]	180 ± 6[f]	539 ± 11[f]	0.33 ± 0.01[f]

[1] BWCh = Body weight change.

[2] C = period to reach BW of 80 ± 5 g.

[3] FI = Food intake.

[4] FER = Food efficiency ratio.

[5] E = 35-day period following C.

[6] Probability of a larger value of the F statistic calculated to test the hypothesis that the group means are equal. (For example, P > F = 0.05 would indicate significance at the 5% level.)

[7] Means ± S.E.

* Statistical analysis: means with same superscripts in a column are not different (P > 0.05).

Table 28. (Continued)

Group	Exp. diet	$BWCh_D$[1,8] g	FI_D[3] g	FER_D[4]	$BWCh_T$[9] g	FI_T g	FER_T
P > F within a column[6]		0.0007	0.0003	0.0038	0.0002	0.0001	0.0159
XVI	5¼ Mg	31 ± 5[h,i,j*]	161 ± 6[f,g,h]	0.19 ± 0.03[g,h,i]	196 ± 10[e]	714 ± 18[e]	0.27 ± 0.01[e]
XVII	5X B_6	35 ± 3[i,j,k]	156 ± 9[e,f,g,h]	0.22 ± 0.02[i]	203 ± 12[e]	682 ± 22[e]	0.30 ± 0.01[f,g,h]
XVIII	5X tryp	22 ± 3[f,h]	137 ± 8[e,f]	0.15 ± 0.02[f,g,h]	189 ± 9[e]	694 ± 30[e]	0.27 ± 0.01[e]
XIX	5¼ Mg+5X B_6	36 ± 5[i,j,k]	167 ± 8[g,h,i]	0.21 ± 0.02[h,i]	212 ± 11[e]	709 ± 25[e]	0.30 ± 0.01[f,g,h]
XX	5X tryp + 5XB_6	20 ± 3[f,h]	133 ± 13[e]	0.15 ± 0.01[f,g,h]	180 ± 21[e]	656 ± 48[e]	0.27 ± 0.01[e]
XXI	5¼ Mg + 5X tryp + 5X B_6	26 ± 2[f,h,i]	153 ± 7[e,f,g,h]	0.17 ± 0.01[g,h]	195 ± 11[e]	700 ± 23[e]	0.28 ± 0.01[e,f]
XXII	[Mn = 5X Mg] + ¼ Mg	16 ± 5[f]	153 ± 13[e,f,g,h]	0.09 ± 0.02[f]	191 ± 15[e]	694 ± 34[e]	0.27 ± 0.01[e]
XXIII	¼ Mg	26 ± 7[f,h,i]	150 ± 11[e,f,g]	0.14 ± 0.05[f,g]	188 ± 17[e]	687 ± 27[e]	0.27 ± 0.02[e]
XXIV	21.5% protein	38 ± 5[j,k]	188 ± 9[i,j]	0.19 ± 0.02[g,h,i]	246 ± 7[f]	852 ± 17[f]	0.29 ± 0.01[e,f,g]
XXV	Control (30% protein)	43 ± 4[k]	193 ± 8[j]	0.22 ± 0.02[i]	259 ± 8[f]	806 ± 16[f]	0.32 ± 0.01[h]

[8] D = 11-day period following E.

[9] T = Total period including C-D.

During the D-period, the 21.5% protein and control groups had greater body weight gains than other groups; differences were significant between controls and all other groups except the 21.5% protein group. The groups fed 21.5% protein (Group XXIV), 5¼ magnesium (XVI), 5X B_6 (XVII) and 5¼ magnesium + 5X B_6 (XIX) had higher weight gains than those recorded for most other groups. Similar patterns were observed for food intakes and food efficiencies.

Adding tryptophan at 5X the NRC requirement to rations in period D tended to depress body weight changes, food intakes to a lesser extent, and food efficiencies (Groups XVIII, XX, XXI as compared to corresponding groups without added tryptophan, XVII, XIX and XXIII). Added manganese tended to depress body weight change and food efficiency for Group XXII as compared with Group XXIII receiving a similar level of magnesium but without added manganese. Food efficiency was low in magnesium deficiency with no supplement (Group XXIII) or with tryptophan or tryptophan and B_6 (XVIII, XX), but equalled that of controls if magnesium and/or vitamin B_6 was added to the diet. Substituting an equivalent weight of manganese for magnesium (Groups XXII, XVI) reduced food efficiency markedly.

Factorial analysis of variance revealed an effect ($P < 0.05$) of the supplements on body weight change during the D-period. Supplementation with vitamin B_6 alone was associated with body weight gain above that of unsupplemented groups; adding B_6 and tryptophan resulted in a lesser body weight gain than that of unsupplemented groups. These

effects were observed for both levels of magnesium. Magnesium intake had no significant effect on weight gain during this period and there was no interaction between magnesium and supplements. Adding magnesium to diets tended to increase food intake during the D-period ($P < 0.09$). The supplements had no effect on food intake and had no interaction with magnesium.

Total body weight changes, food intake and food efficiency reflected the pattern observed during the E-period for these parameters. Groups XVI-XXIII tended to be similar to each other and different from Groups XXIV and XXV. This was expected, since the E-period was slightly more than 3 times as long as the D-period and therefore contributed a correspondingly greater proportion of the total values for each parameter.

Autopsy and organ weights

Autopsy weights (Table 29) also reflected the dietary treatment during the E-period with Groups XVI-XXIII having similar weights which were smaller ($P < 0.01$) than those of Groups XXIV (21.5% protein) and XXV (30% protein). Autopsy weights of the manganese-supplemented Group (XXII) and low-magnesium Group (XXIII) were similar. Supplements produced the same trend in autopsy weight as reported for body weight change in the previous section, but the effect was not significant, probably due to the relatively short feeding period. Groups receiving magnesium during the D-period (XVI, XIX, XXI) tended to be heavier than corresponding groups not receiving magnesium (XXIII, XVII, XX).

Spleen weights (Table 29) among the ten groups were similar. When expressed as percentages of the body weights, spleens from Groups XVI-

Table 29. Mean body and organ weights at autopsy in experiment 6

P > F within a column[1]		Autopsy wt.	Spleen wt.		Kidney wt.	
		0.0001	0.1996	0.0093	0.0037	0.0014
Group	Exp. diet	g	mg	% AWT[2]	g	% AWT
XVI	5¼ Mg	241 ± 10[3,c*]	618.4 ± 31.2[c,d]	0.26 ± 0.01[c,d,e]	2.90 ± 0.13[d,e,f,g]	1.22 ± 0.08[d]
XVII	5X B_6	250 ± 12[c]	707.5 ± 34.0[d,e]	0.28 ± 0.01[d,e,f]	3.06 ± 0.10[f,g]	1.25 ± 0.08[d]
XVIII	5X tryp	235 ± 9[c]	625.5 ± 37.8[c,d,e]	0.27 ± 0.01[d,e,f]	2.89 ± 0.17[d,e,f,g]	1.25 ± 0.09[d]
XIX	5¼ Mg + 5X B_6	258 ± 11[c]	737.8 ± 39.4[e]	0.29 ± 0.01[e,f]	3.18 ± 0.21[g]	1.24 ± 0.08[d]
XX	5X tryp + 5X B_6	226 ± 20[c]	649.1 ± 34.5[c,d,e]	0.30 ± 0.03[f]	2.56 ± 0.21[c]	1.19 ± 0.12[d]
XXI	5¼ Mg + 5X tryp + 5X B_6	239 ± 11[c]	626.1 ± 34.3[c,d,e]	0.26 ± 0.01[c,d,e]	3.04 ± 0.26[f,g]	1.32 ± 0.17[d]
XXII	[Mn = 5X Mg] + ¼ Mg	236 ± 14[c]	583.0 ± 37.6[c]	0.25 ± 0.01[c,d]	2.65 ± 0.09[c,d,e,f]	1.18 ± 0.12[d]
XXIII	¼ Mg	231 ± 17[c]	697.6 ± 77.6[c,d,e]	0.30 ± 0.03[f]	2.72 ± 0.13[d,e,f]	1.24 ± 0.11[d]
XXIV	21.5% protein	293 ± 7[d]	687.6 ± 31.3[c,d,e]	0.23 ± 0.01[c]	2.34 ± 0.07[c]	0.80 ± 0.01[c]
XXV	Control (30% protein)	304 ± 8[d]	706.9 ± 49.2[d,e]	0.23 ± 0.01[c]	2.59 ± 0.06[c,d,e]	0.85 ± 0.02[c]

[1]Probability of a larger value of the F statistic calculated to test the hypothesis that the group means are equal. (For example, P > F = 0.05 would indicate significance at the 5% level.)

[2]AWT = Autopsy weight.

[3]Mean ± S.E.

*Statistical analysis: means with the same superscripts in a column are not different (P > 0.05).

Table 29. (Continued)

		Liver wt.		Testes wt.		Thymus wt.	
P > F within a column[1]		0.0001	0.0001	0.6582	0.0083	0.2578	0.5098
Group	Exp. diet	g	% AWT[2]	g	% AWT	mg	% AWT
XVI	5½ Mg	9.05±0.44[e,f,c,d]	3.75±0.09[e]	3.11±0.10[c,d]	1.30±0.05[e]	363.4±26.9[c]	0.15±0.01[c,d]
XVII	5X B_6	9.20±0.56[c,d,e]	3.65±0.09[d]	3.19±0.04[d]	1.30±0.06[e]	456.8±42.7[c,d,e,f,g]	0.18±0.01[d]
XVIII	5X tryp	8.12±0.37[c,d]	3.45±0.08[c,d]	3.12±0.13[c,d]	1.35±0.08[e]	376.6±32.4[c,d,e,f]	0.16±0.01[c,d]
XIX	5½ Mg+5X B_6	10.44±0.63[e,f]	4.01±0.10[e]	3.10±0.08[c,d]	1.21±0.05[c,d,e]	446.0±44.2[c,d,e,f,g]	0.17±0.01[c,d]
XX	5X tryp+5XB_6	7.66±0.79[c]	3.36±0.12[c]	2.87±0.16[c]	1.31±0.08[e]	416.2±60.7[c,d,e,f,g]	0.18±0.02[d]
XXI	5½ Mg + 5X tryp+5XB_6	9.22±0.41[d,e]	3.86±0.06[e]	3.03±0.07[c,d]	1.29±0.06[e]	370.5±44.8[c,d,e]	0.15±0.02[c,d]
XXII	[Mn = 5X Mg] + ½ Mg	8.28±0.67[c,d]	3.48±0.12[c]	3.01±0.09[c,d]	1.30±0.07[e]	348.7±41.0[c]	0.14±0.01[c]
XXIII	½ Mg	8.86±0.75[c,d]	3.80±0.13[e]	3.10±0.23[c,d]	1.38±0.09[e]	426.2±62.6[c,d,e,f,g]	0.17±0.02[c,d]
XXIV	21.5% protein	11.31±0.49[f]	3.85±0.09[e]	3.13±0.11[c,d]	1.07±0.05[c]	512.9±49.5[g]	0.17±0.01[c,d]
XXV	Control (30% protein)	11.97±0.45[f]	3.93±0.08[e]	3.26±0.03[d]	1.08±0.03[c,d]	452.6±22.0[c,d,e,f]	0.15±0.00[c,d]

XXIII, which had received magnesium-deficient diets during the E-period, were heavier than those from Groups XXIV (21.5% protein) and XXV (controls), which had received adequate magnesium. Supplementation with vitamin B_6 or B_6 and tryptophan had no effect on spleen weight and no consistent trend was apparent. Spleens from animals receiving magnesium during the D-period (Groups XVI and XXI) tended ($P > 0.05$) to be smaller relative to body size than those not receiving magnesium. Group XIX was the exception, but these animals were larger and spleen weights were the same in relation to body weights as those of Group XVII (without magnesium). Manganese supplementation (Group XXII) decreased spleen weight relative to body size in comparison to the low-magnesium group (Group XXIII) ($P < 0.05$), as did addition of a similar weight of magnesium (Group XVI).

Kidneys (Table 29) from Groups XVI-XXIII were generally heavier than those from Group XXIV or XIV which had received adequate magnesium during the entire experiment. Kidneys from the latter 2 groups also were smaller ($P < 0.01$) relative to body weight than those from Groups XVI-XXIII. Neither magnesium nor manganese supplementation affected kidney weight, on either basis, relative to the low magnesium group.

Livers (Table 29) of Groups XXIV and XXV were larger than those of the other 8 groups. Additional magnesium fed during the D-period was associated with larger livers ($P < 0.06$) and also with larger livers relative to body weight ($P < 0.01$). Supplementation with either vitamin B_6 or B_6 and tryptophan had no significant effect on total liver weight although there was a trend toward larger livers for vitamin B_6-supplemented

groups and equal or smaller livers in B_6-tryptophan-supplemented groups at both levels of magnesium. When livers were compared relative to body weight by factorial analysis of variance, a significant interation between magnesium level and supplementation was found ($P < 0.05$). When either organic supplement was given along with magnesium, livers were larger relative to body weight than when magnesium was not added. When no additional magnesium was given, supplementation was associated with smaller livers relative to body weight. Livers from the manganese-supplemented animals (XXII) tended to be smaller than those of magnesium-deficient (XXIII) animals; the difference became significant ($P < 0.05$) when groups were compared on a body weight basis.

Total testes weights (Table 29) among groups were not different, but were larger ($P < 0.01$) relative to body weight in Groups XVI-XXIII fed magnesium-deficient diets during the E-period than in Groups XXIV and XXV.

Thymus weights (Table 29) among groups were not different on either a total weight or relative body weight basis.

Femur weights, measurements, and mineral concentrations

Femur weights in Table 30a refer to the wet weights of the bones and were heaviest in 21.5% protein-fed and control animals. Trends for total femur weight and femur weight relative to body weight were the same as for autopsy weight. Femurs were heavier as percentages of body weights when no magnesium was fed than when 5 times the required amount was added. Magnesium-feeding therefore influenced body weight more than femur weight. Feeding manganese did not affect femur weight.

Table 30a. Femur weights, measurements; manganese and magnesium concentrations at autopsy in experiment 6

		Femur wt.		Femur diameters at positions indicated in Fig. 1			
				D_1	D_2	D_3	D_4
P > F within a column[1]		0.0655	0.0102	0.0001	0.0280	0.7370	0.2363
Group	Exp. diet	mg	% AWT[2]	mm	mm	mm	mm
XVI	5½ Mg	862.7±27.5[3,c*]	0.36±0.01[c,d,e]	5.25±0.08[c]	3.92±0.08[c,d]	2.98±0.07[c]	6.83±0.11[c,d,e]
XVII	5X B_6	883.5±23.8[c,d]	0.36±0.01[c,d,e]	5.25±0.08[c]	4.02±0.11[c,d,e]	3.04±0.06[c]	6.82±0.06[c,d,e]
XVIII	5X tryp	916.7±25.5[c,d,e]	0.39±0.01[e]	5.22±0.07[c]	3.97±0.08[c,d,e]	3.09±0.05[c]	6.82±0.06[c,d,e]
XIX	5½ Mg+5X B_6	887.6±33.0[c,d]	0.35±0.01[c,d]	5.32±0.14[c]	4.00±0.07[c,d,e]	3.05±0.07[c]	6.85±0.07[c,d,e]
XX	5X tryp + 5X B_6	860.5±47.1[c]	0.39±0.02[e]	5.08±0.12[c]	3.80±0.07[c]	2.96±0.06[c]	6.72±0.10[c]
XXI	5½ Mg + 5X tryp + 5X B_6	862.1±36.6[c]	0.36±0.01[c,d,e]	5.17±0.10[c]	3.91±0.08[c]	3.08±0.05[c]	6.80±0.07[c,d,e]
XXII	[Mn = 5X Mg] + ½ Mg	847.4±53.1[c]	0.37±0.03[d,e]	5.12±0.07[c]	3.84±0.08[c]	3.00±0.03[c]	6.72±0.07[c]
XXIII	½ Mg	862.8±42.3[c]	0.39±0.02[e]	5.31±0.09[c]	3.92±0.07[c,d]	2.99±0.05[c]	6.77±0.09[c,d]
XXIV	21.5% protein	970.3±32.5[d,e]	0.33±0.01[c]	5.70±0.18[d]	4.18±0.08[e]	3.05±0.05[c]	7.01±0.08[e]
XXV	Control (30% protein)	992.7±37.0[e]	0.33±0.01[c]	5.75±0.10[d]	4.14±0.08[d,e]	3.07±0.04[c]	6.94±0.08[d,e]

[1]Probability of a larger value of the F statistic calculated to test the hypothesis that the group means are equal. (For example, P > F = 0.05 would indicate significance at the 5% level.)

[2]AWT = Autopsy weight.

[3]Mean ± S.E.

*Statistical analysis: means with same superscripts in a column are not different (P> 0.05).

Table 30a. (Continued)

Group	Exp. diet	Femur length	Ash wt.	Mn/ femur ash	Femur Mn/AWT	Mg/ femur ash	Femur Mg/AWT
P > F within a column[1]		0.0037	0.0232	0.0805	0.0001	0.0001	0.0001
		mm	mg	ppm	ppm	ppm	ppm
XVI	5¼ Mg	33.2±0.3[3,c*]	255.3±9.7[c]	11.3±0.3[c]	0.012±0.000[d,e]	5591±261[d]	6.0±0.3[f,g,h]
XVII	5X B_6	33.4±0.4[c]	259.2±9.4[c]	11.3±0.4[c]	0.012±0.000[d,e]	3093±153[c]	3.2±0.2[c]
XVIII	5X tryp	33.8±0.4[c,d]	266.1±9.6[c,d]	11.6±0.5[c]	0.013±0.000[e,f]	3104±140[c]	3.5±0.1[c]
XIX	5¼ Mg + 5X B_6	33.7±0.4[c]	260.7±11.6[c,d]	10.8±0.6[c]	0.011±0.000[c]	5468±501[d]	5.5±0.5[f]
XX	5X tryp+5X B_6	32.8±0.7[c]	256.6±20.7[c]	12.4±0.6[d]	0.014±0.000[f]	3340±119[c]	3.8±0.2[c,d,e]
XXI	5¼ Mg + 5X tryp + 5X B_6	33.0±0.5[c]	252.9±13.1[c]	11.5±0.4[c]	0.012±0.000[d,e]	5524±441[d]	5.8±0.5[f,g]
XXII	[Mn = 5X Mg] + ¼ Mg	33.4±0.5[c]	266.3±10.2[c,d]	12.0±0.4[d]	0.014±0.000[f]	3279±112[c]	3.8±0.2[c,d,e]
XXIII	¼ Mg	32.9±0.5[c]	260.4±13.4[c,d]	11.6±0.4[c,d]	0.013±0.000[e,f]	3477±274[c]	4.1±0.5[d,e]
XXIV	21.5% protein	34.9±0.3[d]	291.3±7.2[d,e]	10.5±0.3[c]	0.010±0.000[c]	6821±279[e]	6.8±0.3[h]
XXV	Control (30% protein)	34.9±0.2[d]	304.6±8.1[e]	10.7±0.4[c]	0.011±0.000[c,d]	6696±172[e]	6.7±0.2[h]

Femur diameter at positions D_1 (Fig. 1) were larger in controls and 21.5% protein-fed animals than in all other groups ($P < 0.01$) which had been depleted of magnesium in the E-period. Similarly, at position D_2 diameters in Groups XXIV and XXV were larger than in most other groups. Femurs were also significantly longer in the latter 2 groups.

Ash weight was positively correlated with femur weight. As expected, femur ash was highest in control and 21.5% protein-fed animals.

There were no differences among groups for manganese concentration in femur ash. Even manganese feeding (Group XXII) had no effect on femur manganese compared to the low-magnesium group (XXIII), though it resulted in higher content of manganese than in rats fed a similar amount of magnesium (XVI). Variations in femur manganese concentration on a body weight basis were functions of body size, with larger animals having less femur manganese relative to body size.

Femur magnesium content was dependent upon dietary magnesium ($P < 0.01$). Bone served as a reserve for magnesium (XXV) and became depleted when the diet was deficient (XXIII), but repletion occurred rapidly upon refeeding in this experiment (XVI). Groups receiving magnesium had less magnesium ($P < 0.01$) than controls and 21.5% protein-fed animals but more ($P < 0.01$) than similar groups not fed magnesium.

Significant differences ($P < 0.05$) among groups in ratio of femur length to diameter at position D_1 (FL/D_1) existed (Table 30b). The only pattern which emerged was the result of diameters at D_1 which tended to be larger for the control group and 21.5% protein group than for the other groups. The magnesium-deficient group (XXIII) had the same low ratio of

femur length to diameter at D_1 as these groups, but it was because femurs were short in Group XXIII (Table 30a). Magnesium levels and other supplements had no effect on FL/D_1 values. Femurs were long relative to diameter at D_3 (FL/D_3) in control and 21.5% protein-fed animals. No other consistent pattern of group differences was apparent.

Diameter-diameter ratios D_1/D_3 and D_2/D_3 were different among groups but again the differences seemed to lie between groups deprived of magnesium during the E-period (Groups XVI-XXIII) and those which received adequate magnesium (Groups XXIV and XXV). Groups XXIV and XXV had higher ratios for both D_1/D_3 and D_2/D_3 because of larger diameters at D_1 and D_2 relative to D_3, which did not change with diet. The sums of the bone diameters ($D_{1+2+3+4}$) were greater for Groups XXIV and XXV as compared to groups which had been deprived of magnesium, largely because of the smaller diameters at D_1 and D_2 (Fig. 1) in magnesium deficiency. Neither manganese supplementation, magnesium level, vitamin B_6 nor B_6-tryptophan supplementation had effects on relative bone dimensions in this experiment.

Splenic nucleic acids

Spleen homogenates were analyzed for RNA and DNA content (Table 31). There were no differences among groups for either total RNA and DNA or concentration of either. Neither magnesium level, manganese supplementation or feeding additional B_6 or B_6-tryptophan affected nucleic acids in the spleen.

Table 30b. Ratios of femur length (FL) to bone diameters (D) and ratios of bone diameters to each other (D/D) at the positions indicated in Figure 1, experiment 6

P > F within[1] a column		FL/D_1 0.0367	FL/D_2 0.7446	FL/D_3 0.0356	FL/D_4 0.1594	D_4/D_1 0.0093	D_4/D_2 0.1812	D_4/D_3 0.5066	D_1/D_2 0.4041	D_1/D_3 0.0002	D_2/D_3 0.0095	$D_{1+2+3+4}$ 0.0046
Group	Exp. diet	mm/mm	mm/mm	mm/mm	mm/mm	mm/mm	mm/mm	mm/mm	mm/mm	mm/mm	mm/mm	mm
XVI	5¼ Mg	$6.3^{a,b*}$	8.5^{a}	$11.2^{b,c,d,e}$	4.9^{a}	1.30^{c}	$1.74^{a,b}$	2.30^{b}	$1.34^{a,b}$	$1.77^{b,c,d}$	$1.32^{a,b,c}$	18.98^{a}
XVII	5X B_6	6.4^{b}	8.4^{a}	$11.0^{a,b,c}$	4.9^{a}	1.30^{c}	$1.71^{a,b}$	$2.25^{a,b}$	1.31^{a}	$1.73^{a,b,c,d}$	$1.32^{a,b,c}$	19.12^{a}
XVIII	5X tryp	6.5^{b}	8.5^{a}	$10.9^{a,b}$	4.9^{a}	1.31^{c}	$1.72^{a,b}$	2.21^{a}	1.32^{a}	$1.69^{a,b}$	1.28^{a}	19.09^{a}
XIX	5¼Mg+5XB_6	$6,4^{b}$	8.4^{a}	$11.1^{b,c,d}$	4.9^{a}	1.30^{c}	$1.72^{a,b}$	$2.25^{a,b}$	$1.33^{a,b}$	$1.74^{a,b,c,d}$	$1.31^{a,b}$	19.21^{a}
XX	5Xtryp+5XB_6	6.4^{b}	8.6^{a}	$11.1^{b,c,d}$	4.9^{a}	1.32^{c}	1.77^{b}	$2.28^{a,b}$	$1.34^{a,b}$	$1.72^{a,b,c}$	1.29^{a}	18.56^{a}
XXI	5¼ Mg + 5X tryp + 5XB_6	6.4^{b}	8.5^{a}	10.7^{a}	4.9^{a}	1.32^{c}	$1.74^{a,b}$	2.21^{a}	$1.33^{a,b}$	1.68^{a}	1.27^{a}	18.96^{a}
XXII	[Mn = 5X Mg] + ¼ Mg	6.5^{b}	8.7^{a}	$11.1^{b,c,d}$	5.0^{b}	1.31^{c}	$1.75^{a,b}$	$2.24^{a,b}$	$1.33^{a,b}$	$1.71^{a,b,c}$	1.28^{a}	18.68^{a}
XXIII	¼ Mg	6.2^{a}	8.4^{a}	$11.0^{a,b,c}$	4.9^{a}	$1.28^{b,c}$	$1.73^{a,b}$	$2.27^{a,b}$	$1.35^{a,b}$	$1.78^{c,d}$	$1.31^{a,b}$	18.99^{a}
XXIV	21.5% protein	6.2^{a}	8.4^{a}	11.5^{e}	5.0^{b}	$1.24^{a,b}$	1.68^{a}	2.30^{b}	$1.36^{a,b}$	1.87^{e}	1.37^{c}	19.94^{b}
XXV	Control (30% protein)	6.1^{a}	8.5^{a}	$11.4^{d,e}$	5.0^{b}	1.21^{a}	1.68^{a}	$2.26^{a,b}$	1.39^{b}	1.87^{e}	$1.35^{b,c}$	19.91^{b}

[1]Probability of a larger value of the F statistic calculated to test the hypothesis that the group means are equal. (For example, P > F = 0.05 would indicate significance at the 5% level.)

*Statistical analysis: means with the same superscripts in a column are not different (P > 0.05).

Table 31. Mean nucleic acid content of spleens in experiment 6

Group	Exp. Diet	Total RNA	mg RNA/ g spleen	Total DNA	mg DNA/ g spleen	RNA/ DNA
P > F within a column[1]		0.2855	0.3563	0.5676	0.7201	0.6061
		mg		mg		
XVI	5¼ Mg	3.41 ± 0.25[2,b,c,d,*]	5.48 ± 0.17[b,c]	13.21 ± 1.21[b]	21.11 ± 0.94[b,c]	0.26 ± 0.01[b,c,d]
XVII	5X B_6	3.88 ± 0.23[b,c,d]	5.47 ± 0.15[b,c]	15.56 ± 0.62[b]	22.15 ± 0.66[b,c]	0.25 ± 0.01[b,c]
XVIII	5X tryp	3.02 ± 0.22[b,c]	4.82 ± 0.13[b]	13.01 ± 0.87[b]	20.89 ± 0.96[b,c]	0.24 ± 0.02[b]
XIX	5¼ Mg + 5X B_6	3.93 ± 0.31[b,c,d]	5.27 ± 0.16[b,c]	15.09 ± 1.08[b]	20.37 ± 0.89[b,c]	0.26 ± 0.01[b,c,d]
XX	5X tryp + 5X B_6	3.32 ± 0.19[b,c,d]	5.13 ± 0.16[b,c]	13.42 ± 1.02[b]	20.60 ± 0.88[b,c]	0.25 ± 0.01[b,c]
XXI	5¼ Mg + 5X tryp + 5X B_6	3.41 ± 0.26[b,c,d]	5.42 ± 0.16[b,c]	13.61 ± 1.31[b]	21.52 ± 1.27[b,c]	0.26 ± 0.01[b,c,d]
XXII	[Mn=5XMg]+¼ Mg	3.01 ± 0.19[b]	5.17 ± 0.11[b,c]	12.14 ± 1.11[b]	20.83 ± 1.35[b,c]	0.26 ± 0.01[b,c,d]
XXIII	¼ Mg	4.18 ± 0.68[d]	5.75 ± 0.43[c]	15.82 ± 1.98[b]	22.71 ± 1.44[c]	0.26 ± 0.02[b,c,d]
XXIV	21.5% protein	3.80 ± 0.40[b,c,d]	5.45 ± 0.35[b,c]	13.28 ± 0.88[b]	19.26 ± 0.74[b]	0.29 ± 0.02[d]
XXV	Control (30% protein)	4.08 ± 0.65[c,d]	5.57 ± 0.38[c]	15.23 ± 2.50[b]	20.88 ± 1.72[b,c]	0.27 ± 0.01[b,c,d]

[1]Probability of a larger value of the F statistic calculated to test the hypothesis that the group means are equal. (For example, P > F = 0.05 would indicate significance at the 5% level.)

[2]Mean ± S.E.

*Statistical analysis: means with the same superscripts in a column are not different (P > 0.05).

Antibody titers and gamma globulin fractions

Agglutinin and hemolysin titers were determined 5 and 9 days following immunization with sheep red blood cells. Analysis of variance revealed no differences among groups for agglutinin levels, hemolysin levels or for any of the gamma globulin parameters. Factorial analysis of variance of 6 groups revealed some differences in serum antibody titers related to magnesium level and to supplementation of diets with vitamin B_6 or B_6-tryptophan, however.

On day 5, lower agglutinin values ($P < 0.06$) were observed when diets were supplemented with B_6 or B_6-tryptophan (Groups XVII, XIX, XX, XXI) at either level of magnesium than for the corresponding unsupplemented groups (XVI, XXIII) (Table 32). The same trend was seen on day 9 but the effect was not significant. Magnesium level possibly affected agglutinin values for day 9, with Groups XVI, XIX, and XXI (magnesium-fed) having higher titers ($P < 0.08$) than corresponding Groups XVII, XX, and XXIII which did not receive magnesium. The same pattern was observed on day 5 but those differences also were not significant. Manganese feeding (Group XXII) tended to lower agglutinin titers on both days below those for magnesium-deficient animals (Group XXIII) ($P < 0.05$) and those given a similar amount of magnesium (Group XVI) ($P < 0.05$ on day 5).

Hemolysin values were lower in animals receiving vitamin B_6 or B_6-tryptophan supplements at both levels of magnesium (Groups XIX, XXI, XVII, XX) on day 5 ($P < 0.06$) and on day 9 ($P < 0.05$) than in

corresponding unsupplemented groups. Magnesium deficiency was associated with lower hemolysin titers on both days but the effect was not significant. Added manganese (Group XXII) tended to lower hemolysin titers on both days compared to magnesium-deficient animals (Group XXIII), but the differences were not significant.

The concentrations of total gamma globulin and of 19S and 7S subfractions were generally similar for sera which were analyzed: control and magnesium-deficient groups and those given a single supplement of magnesium, manganese, vitamin B_6 or tryptophan. The only effect was a relatively high total protein value for manganese-fed rats, which had the highest concentration for each component measured.

Table 32. Mean serum antibody titers 5 and 9 days following immunization and serum gamma globulin fractionation data in experiment 6

		Agglutinin		Hemolysin		Protein
		Day 5	Day 9	Day 5	Day 9	G.G. ext.[2,3]
P > F within a column[1]		0.3557	0.4256	0.1748	0.1493	0.8194
Group	Exp. diet	log titer		log titer		mg/100 ml
XVI	5¼ Mg	2.91 ± 0.33[4,g*]	3.29 ± 0.10[f]	2.48 ± 0.36[h]	2.50 ± 0.27[g]	23.94 ± 4.57[e]
XVII	5X B_6	1.82 ± 0.35[e]	2.50 ± 0.23[e]	1.63 ± 0.21[e]	1.78 ± 0.12[e]	28.20 ± 2.94[e]
XVIII	5X tryp	2.10 ± 0.32[e,f,g]	2.89 ± 0.13[e,f]	1.92 ± 0.14[e,f,g,h]	2.12 ± 0.18[e,f,g]	29.99 ± 4.43[e]
XIX	5¼ Mg + 5X B_6	2.14 ± 0.29[e,f,g]	3.19 ± 0.14[f]	2.02 ± 0.14[e,f,g,h]	2.19 ± 0.19[e,f,g]	----
XX	5X tryp + 5X B_6	2.39 ± 0.23[e,f,g]	2.90 ± 0.25[e,f]	1.77 ± 0.17[e,f,g]	2.04 ± 0.09[e,f,g]	----
XXI	5¼ Mg + 5X tryp + 5X B_6	2.60 ± 0.33[e,f,g]	2.82 ± 0.24[e,f]	2.03 ± 0.28[e,f,g,h]	2.10 ± 0.17[e,f,g]	----
XXII	[Mn = 5X Mg] + ¼ Mg	2.06 ± 0.25[e,f]	2.78 ± 0.17[e,f]	1.70 ± 0.08[e,f]	1.93 ± 0.10[e,f]	33.03 ± 4.74[e]
XXIII	¼ Mg	2.59 ± 0.35[e,f,g]	3.01 ± 0.27[e,f]	2.28 ± 0.25[f,g,h]	2.35 ± 0.22[f,g]	31.66 ± 6.23[e]
XXIV	21.5% protein	2.69 ± 0.33[f,g]	2.85 ± 0.28[e,f]	2.20 ± 0.24[e,f,g,h]	1.96 ± 0.09[e,f]	----
XXV	Control (30% protein)	2.46 ± 0.29[e,f,g]	2.81 ± 0.27[e,f]	2.09 ± 0.18[e,f,g,h]	2.21 ± 0.17[e,f,g]	29.18 ± 3.17[e]

[1]Probability of a larger value of the F statistic calculated to test the hypothesis that the group means are equal. (For example, P> F = 0.05 would indicate significance at the 5% level.)

[2]G.G. Ext. = Gamma globulin extract.

[3]Total protein values for 7S + 19S + Other were determined on solutions 2.5X more concentrated than solutions on which G.G. ext. protein values were measured.

[4]*Mean ± S.E.

*Statistical analysis: means with the same superscripts in a column are not different (P>0.05).

Table 32. (Continued)

Group	Exp. diet	Total protein (7S+19S+ other) [3]	Protein 19S	Protein 19S	Protein 7S	Protein 7S
P > F within a column [1]		0.1792	0.6155	0.9080	0.3758	0.9832
		mg/100 ml	mg/100 ml	%T [5]	mg/100 ml	%T
XVI	5½ Mg	47.52 ± 6.21[4,e,f,g*]	4.90 ± 1.24[e]	10.80 ± 2.81[e]	32.04 ± 6.32[e]	65.40 ± 7.91[e]
XVII	5X B_6	44.57 ± 5.36[e,f,g]	6.80 ± 2.34[e]	13.22 ± 2.95[e]	26.50 ± 2.99[e]	60.11 ± 3.75[e]
XVIII	5X tryp	45.47 ± 7.88[e,f,g]	6.97 ± 1.39[e]	15.89 ± 2.04[e]	27.87 ± 5.13[e]	60.56 ± 3.96[e]
XIX	5½ Mg + 5X B_6	----	----	----	----	----
XX	5X tryp + 5X B_6	----	----	----	----	----
XXI	5½ Mg + 5X tryp + 5X B_6	----	----	----	----	----
XXII	[Mn = 5X Mg] + ½ Mg	57.71 ± 7.29[g]	9.33 ± 2.75[e]	13.50 ± 2.68[e]	33.80 ± 3.89[e]	60.00 ± 2.91[e]
XXIII	½ Mg	39.49 ± 4.23[e,f]	5.39 ± 1.33[e]	15.44 ± 4.38[e]	24.83 ± 4.10[e]	59.67 ± 6.25[e]
XXIV	21.5% protein	----	----	----	----	----
XXV	Control (30% protein)	36.60 ± 4.43[e]	5.29 ± 1.41[e]	13.10 ± 2.21[e]	22.39 ± 3.49[e]	60.50 ± 4.99[e]

[5] %T = % of total protein (7S + 19S + Other).

DISCUSSION

Protein synthesis is an intracellular process which requires, among other factors, proper amounts of amino acids, a source of energy, ribosomes, and nucleic acids. It follows that changes in the supply of or modifications which influence the utilization of the factors involved in protein synthesis can affect the quantity of protein produced, the rate at which it is synthesized, and possibly the type of protein generated. Although various homeostatic mechanisms function to maintain a constant intracellular environment, changes in it and in the external environment occur which can influence cellular metabolism.

It is obvious from the results of this study that lack of dietary magnesium produced a marked difference in cellular metabolism as assessed by several parameters; the depression of serum antibody titers was of particular significance. The synthesis of antibodies from free amino acids (Schoenheimer et al., 1942) has been shown to follow the general pattern of mammalian protein synthesis (Haurowitz, 1965). The role of magnesium in the synthesis of protein, therefore, assumes increased significance. Participation by magnesium in the control of cell mutation and cell division has also been suggested. Bois (1964) postulated that a chronic lack of cellular magnesium could increase the movement of magnesium from the nucleus into the cytoplasm, thereby causing chromosomal aberrations and cell mutations. Battifora et al. (1968) advanced a related hypothesis to explain the leukemia occurring in magnesium-deficient rats, indicating intracellular magnesium deficiency and its possible interference with DNA replication during cell division.

They did not find abnormal chromosomes in their rats, however.

It has been difficult to relate directly a cellular deficiency of magnesium to decreased protein synthesis in specific tissues such as the liver (Schwartz et al., 1969); "magnesium may be preferentially preserved in tissues and cellular sites of active protein synthesis, perhaps at the expense of other metabolic processes which also require magnesium." The influence of magnesium on ribosomal aggregation with the consequent effects on protein biosynthesis has been well documented in vitro. Even so, Schwartz et al. (1970) showed that polysome profiles in livers from magnesium-deficient rats were similar to those from control animals and that cellular magnesium concentration did not change with deficiency under the conditions of their experiment (36% casein diet with 0.008% magnesium for 5 weeks). Intracellular magnesium can be changed in dietary deficiency, however. Magnesium levels in skeletal and cardiac muscle were decreased when a magnesium-deficient diet (0.01%) was fed to guinea pigs for 5 weeks (Grace and O'Dell, 1970), but extracellular concentrations were more severely depressed than intracellular levels. Possibly a sufficient quantity remains in magnesium deficiency for intracellular maintenance of nucleic acid structures, aggregation of ribosomes, and enzyme activation so that protein synthesis may not be affected. As a cofactor for oxidative phosphorylation, magnesium may influence protein synthesis through regulation of energy for peptide bond formation.

Manganese has been reported to facilitate amino acid transport (Pal and Christensen, 1959), as well as to affect immune responses (Cotzias, 1962; Hitchings and Falco, 1946; Hitchings et al., 1949;

Ermenkova and Ermenkov, 1967; Antonova et al., 1968).

Tryptophan may also be a controlling factor in protein synthesis, possibly through regulation of polyribosome aggregation (Hori et al., 1967; Swan et al., 1971; Sidransky et al., 1968, 1971; Baliga et al., 1968; Munro, 1968) or at the induction level of enzyme formation (Henderson, 1970). Several studies have demonstrated that tryptophan concentration of the diet may influence immune response (Axelrod, 1953; Kenney et al., 1970; Gershoff et al., 1968).

Vitamin B_6 may participate in protein synthesis by regulating nucleic acid synthesis, incorporation of amino acids into protein, and polysome aggregation (Axelrod and Trakatellis, 1964); Axelrod, 1953; Gershoff et al., 1968; Trakatellis and Axelrod, 1964, 1965; Montjar et al., 1965; Kumar and Axelrod, 1968).

Several parameters, to be discussed further, were chosen to evaluate specific effects and interactions of these 4 factors in cellular metabolism and protein synthesis.

Body Weight Change, Food Intake, and Food Efficiency

The greatest effect on weight change over all experiments was caused by magnesium. Magnesium-deficient animals consistently gained significantly less weight than animals receiving adequate magnesium. Some animals even lost weight. Decreased weight gain was always associated with a smaller food intake than for animals fed adequate magnesium. The lower food intake did not explain the entire effect of deficiency on weight gain, however, because food efficiencies were also lower. Feeding magnesium to depleted rats during the last period in

experiment 6 increased weight gain, food intake and food efficiency. Magnesium participates in many reactions of consequence in energy metabolism, such as those involving thiamine pyrophosphate, oxidative phosphorylation and glucose metabolism via the Embden-Meyerhoff and glycolytic pathways, in addition to its postulated role in maintaining the structures of macro-molecules of protein synthesis. A dietary deficiency of magnesium therefore may limit the degree to which nutrients can be utilized in the tissue synthesis associated with body weight gain.

Manganese affected weight change significantly only in experiment 3, where animals fed the manganese-deficient diet gained less weight than controls, and in experiment 1, where 3 times the required manganese gave greater gain than the lowest manganese intake. In all experiments, rats fed the manganese-deficient diets tended to be smaller than other groups, and those fed manganese in excess of NRC requirement tended to be larger when it was included without comparable additions of other minerals. Manganese is thought by some (Cotzias, 1962) to participate in reactions of oxidative phosphorylation. In addition, it may facilitate amino acid transport (Pal and Christensen, 1959). It seems possible that extremes of manganese intakes, through small changes in tissue manganese concentrations, could stimulate or retard these reactions so that tissue synthesis and thus weight change could be affected. Since the trend toward greater weight gain was not apparent in animals fed high levels of manganese along with other minerals, perhaps less manganese was absorbed from the gut or available for enzymatic cofactor roles because of competition from the other minerals. It is well known that heavy dietary loads with compounds

of one element can interfere with the utilization of others; manganese deficiency has been generated in birds in the presence of adequate dietary manganese when the diets were also high in iron or calcium compounds (Cotzias, 1962).

Addition of tryptophan in experiment 6 at the level of 5 times the NRC requirement depressed growth, food intake and food efficiency. The tolerance for tryptophan is reportedly 10 to 16 times the requirement (Harper et al., 1970), so the level fed was not expected to be toxic. Other investigators (Russell et al., 1952) suggested that the more complex amino acids are the most toxic because of the number of their metabolic interrelationships, resulting in a variety of biologically active products. Even so, this likely had a negligible effect because of the high concentration of protein fed. Animals fed a high-protein diet generally have a much greater tolerance for amino acids fed in excess because of the rapid growth rate caused by increasing quantities of limiting amino acids and increased activity of the enzymes of amino acid degradation, resulting in rapid removal of the excess amino acids so that plasma amino acid concentrations return to normal (Harper et al., 1970).

In diets low in magnesium, adding vitamin B_6 to the basal quantity of the vitamin slightly increased weight gain. Thus the NRC level may not have met vitamin B_6 needs of rats fed 30% protein in this study.

Other factors affecting weight change, food intake and food efficiency were the sources of dietary carbohydrate and protein. The cornstarch used in experiment 1 was replaced by dextrose in experiment 2. Protein levels were 15% in both experiments. Yellow corn provided

some of the protein in experiment 1, while casein supplemented with methionine was the sole source of protein in experiment 2 and subsequent experiments. Animals in the control and manganese-deficient groups of experiment 2 grew similarly and at a much slower rate than those in experiment 1. Food intake and efficiency were also lower. These observations are in agreement with the findings of others who substituted sugars for cornstarch or dextrin as the source of carbohydrate (Wiener et al., 1963; Harper and Katayama, 1953; Harper and Spivey, 1958; Spivey et al., 1958; Allen and Leahy, 1966). It is possible that the osmotic effects of different carbohydrates in the intestine help to determine food intake and utilization of protein. These studies indicated an inverse relationship between the capacity of dietary carbohydrate to exert osmotic pressure and the food intake and rate of gain of rats. Possibly diets containing cornstarch (lower osmotic effect) passed through the intestine more slowly than dextrose, thereby allowing improved digestion and absorption than in experiment 2. It is also possible that there was decreased amino acid catabolism in the animal body with cornstarch because of prolonged absorption of glucose from it. Introducing a different amino acid pattern into the diet when casein became the sole source of protein may also have influenced growth and food utilization. At any rate, growth rate was improved when protein level was increased to 30% in experiment 3 with dextrose as the carbohydrate source, indicating that protein may have been the limiting factor in the 15% diets when dextrose was fed. A direct comparison of 21.5% and 30% protein in experiment 6 failed to show any effects of protein on parameters measured except for a slightly increased total

FER and reduced thymus weight with 30% protein.

Organ Weights

Generally, organ weights were related to weights of the animals from which they were taken, with heavier animals having proportionately heavier organs. The major exceptions to this were the magnesium-deficient groups, which usually had significantly heavier kidneys, testes and spleens than those which received adequate magnesium.

The increased weight of the kidney was due to deposition of what have been reported to be calcium salts (Aikawa and David, 1969); renal calcium may increase to as much as 15 times normal values in magnesium deficiency and may be controlled by parathyroid hormone (Aikawa, 1971).

Spleens were greatly enlarged in magnesium-deficient animals. The increased weight may have been due to great infiltration of the spleen and other hematopoietic organs with immature granulocytes, as reported by other investigators (McCreary et al., 1967). Nucleic acids were determined on spleens from animals in experiments 3-6. The magnesium-deficient groups usually had somewhat greater total amounts than other groups, but when values were expressed as tissue concentrations, no differences among groups in any of the experiments were demonstrated. Although dietary magnesium, manganese and tryptophan may affect synthesis and stability of nucleic acids, these experiments showed no effect on their total quantities. Deficiency of vitamin B_6 has led to reduced numbers of relatively large cells, possibly due to slow DNA replication (Trakatellis and Axelrod, 1964), but in experiment 6, vitamin B_6 beyond a nearly adequate intake did not affect these

parameters.

Size of the testes was difficult to account for, although they appeared somewhat edematous. Grace and O'Dell (1970) found increased extracellular and intracellular sodium levels with accompanying increases in water in skeletal muscle from magnesium-deficient rats. It is possible that the same condition occurred in the testes.

Femur Weights, Measurements, and Mineral Concentrations

Generally, femur weight and length were related to the weight of the animals from which they were taken, with larger animals having proportionately heavier and longer bones. Dietary manganese had little effect on concentration of manganese in the femur. Conversely, dietary magnesium was positively correlated with femur magnesium concentrations. The fact that femur magnesium rose to approach control concentrations after only 9 days of refeeding is further evidence that bone constitutes a major magnesium reserve. The relation between the concentrations of magnesium in bone and in the diet agrees with the findings of other investigators (Smith and Nisbet, 1968).

Femur diameters at the proximal position D_1 (Figure 1) were small, across all experiments, in animals receiving inadequate magnesium. Smith and Nisbet (1968) noted marked osteoporosis and complete cessation of growth in the proximal end of the tibia of magnesium-deficient rats. Furthermore, Bussell et al. (1972) found unusually sharply defined proximal epiphyseal plates in femurs from magnesium-deficient rats by Grenz ray examination. These observations suggest that magnesium deficiency may have a specific effect on cells in the proximal ends of

long bones.

Antibody Titers and Gamma Globulin Fractions

Magnesium deficiency caused the greatest effect on antibody titers, usually lowering both hemolysin and agglutinin values on any of the stated days after immunization, compared to responses of control animals. In experiment 3, magnesium seemed to cause a delay in reaching maximum agglutinin and hemolysin levels. There was no evidence for a difference in the time at which maximum antibody titers were attained in the older animals of experiment 4, however.

The low antibody titers associated with magnesium deficiency could have reflected either a small amount of antibody protein synthesized or a low serological activity of antibody in the deficiency state. In experiment 4, 7S concentration was higher in control animals than in deficient ones, supporting the findings of Alcock and Shils (1973) that magnesium was required for maintenance of normal levels of 7S antibody. Protein values for the 7S subfraction of gamma globulin were the same when controls and magnesium-deficient animals were compared in experiment 3. In seeming contradiction to the first hypothesis, total gamma globulin concentrations tended to be high in magnesium-deficient animals. McCreary et al. (1966) suggested that even if normal amounts of antibody were produced, its activity might be altered by low concentrations of magnesium in tissues, since magnesium is required for complement fixation in vitro (Levine et al., 1953). This might appear to be another possible explanation for low hemolysin values observed in magnesium deficiency, except that the sera had been diluted with magnesium-containing buffer

before antibody was titrated. Effects of the magnesium-deficient diet could not be attributed to reduced protein intake due to partial inanition; rats fed 21.5% protein ad libitum, about the same amount of protein consumed by magnesium-deficient rats, produced as much antibody as controls fed 30% protein.

High manganese intake significantly lowered the day 14 hemolysin activity in experiment 1 compared with the low manganese group. Similarly, in experiment 6, feeding excess manganese tended to lower agglutinin titers on days 5 and 9 below those for magnesium-deficient animals or those given a weight of magnesium equivalent to the manganese fed. These findings support the work of Hitchings et al. (1949) which identified manganese as a dietary factor which promoted the susceptibility of mice to pneumococcal infection with progressively higher intakes.

Day 5 hemolysin titers were also reduced in the group fed the mixture of minerals at 5 times adequate levels in experiment 5. It is possible that an increase in several minerals may have reduced manganese absorption, competed with it in enzymic reactions, or stimulated more rapid catabolism of this initially-appearing antibody by acting as a cofactor for enzymic degradation.

In experiment 6, supplementation of the diets with vitamin B_6 or B_6-tryptophan at either high or low magnesium intake lowered agglutinin and hemolysin titers compared to those found in corresponding unsupplemented groups. Turnover of protein is more rapid when amino acid supply is abundant. Since tryptophan is often the amino acid present in least supply in the metabolic pool of free amino acids, it is possible that increasing the level at which it is fed supports a faster rate of

turnover. Vitamin B_6 participates in many of the reactions of protein catabolism and synthesis, and of tryptophan catabolism, and may thereby also stimulate protein catabolism in general when present in abundance.

SUMMARY

This study encompassed 6 discrete experiments to investigate: 1) the effect of variations in dietary magnesium and manganese, either the level fed or the ratio of these to other key mineral elements fed, on the immune response of young, male rats to sheep red blood cells and 2) interrelationships in magnesium, manganese, tryptophan, and vitamin B_6 metabolism in magnesium-depleted rats and the effects of these interrelationships on the immune responses of young rats.

Rats were immunized with sheep red blood cells, bled and sacrificed at intervals between days 5 and 14 after immunization. To evaluate the effects of the dietary variables on the immune response, serum agglutinins and hemolysins and total serum gamma globulin were estimated by titration and by absorbancy measurements. Splenic RNA and DNA were determined to assess capacity of the spleen for protein synthesis, since it is a site for gamma globulin and antibody production. Femur concentrations of magnesium and manganese were assessed to provide an index of the nutritional status of animals in relation to these minerals.

Magnesium deficiency depressed growth, antibody titers, femur length and diameter at the proximal end, and femur magnesium concentration; it increased spleen, kidney and testes weights and gamma globulin concentration, but had no effect on splenic nucleic acids.

Manganese intake affected weight gain significantly in only 2 of 5 experiments, influenced antibody titers only on day 14 of experiment 1 and day 5 of experiment 5, and had no effect on organ weights, femur dimensions or composition, or hepatic or splenic nucleic acids.

Tryptophan supplementation depressed growth, food intake, food efficiency and agglutinin and hemolysin titers compared to values for corresponding unsupplemented groups.

Vitamin B_6 supplementation was associated with greater weight gain but lower agglutinin and hemolysin titers than those found in the corresponding unsupplemented groups.

There were no significant interactions of magnesium with either vitamin B_6 or B_6-tryptophan combination except for liver weight, which responded to tryptophan and/or vitamin B_6 only when additional magnesium was fed.

This study indicated that, of the dietary variables investigated, magnesium was most effective in influencing the immune response as evaluated by the parameters discussed.

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